AD_____

Award Number: DAMD17-97-1-7019

TITLE: Mechanism of p53 dependent Apoptosis and its Role in Breast Cancer Therapy

PRINCIPAL INVESTIGATOR: Xinbin Chen, Ph.D.

CONTRACTING ORGANIZATION: Medical College of Georgia
Augusta, Georgia 30912-4810

REPORT DATE: July 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20010403 030

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2000	Annual (1 Jul 99 - 30 Jun 00)

4. TITLE AND SUBTITLE
Mechanism of p53 dependent Apoptosis and its Role in Breast Cancer Therapy

5. FUNDING NUMBERS
DAMD17-97-1-7019

6. AUTHOR(S)
Xinbin Chen, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Medical College of Georgia
Augusta, Georgia 30912-4810

E-MAIL:
xchen@mail.mcg.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

The p53 protein contains several functional domains necessary for inducing cell cycle arrest and apoptosis, that is, activation domain 1 and 2, the proline-rich domain, and the C-terminal basic domain. To further determine the role of each p53 functional domain, we found that deletion of, or mutation at, activation domain 2 abrogates the apoptotic activity but not cell cycle arrest. We also found that at least two of the three domains, that is, activation domain 1 and 2 and the proline-rich domain, are necessary for cell cycle arrest. Interestingly, we found that deletion of activation domain 1 alleviates the requirement of the C-terminal basic domain for apoptotic activity. Furthermore, we found that activation domain 2 and the proline-rich domain form an activation domain for inducing pro-apoptotic genes and the C-terminal basic domain is required for maintaining this activation domain competent for transactivation.

As an extension of our studies to determine how p53 functions as a tumor suppressor, we identified two novel p53 target genes, Dickkopf-1 (Dkk-1), an antagonist of the Wnt oncogenic pathway, and TAP1, a transporter of major histocompatibility class I antigens. This suggests that p53 may suppress transformation by Wnt and play a role in immunosurveillance.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	61
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

_X_ Where copyrighted material is quoted, permission has been obtained to use such material.

_X_ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

_X_ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

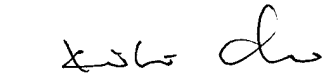
N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

X In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

X In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.



PI - Signature

7-20-00

Date

Table of Contents

1. **Chen, X.** 1999. The p53 family: same response, different signals? ***Molecular Medicine Today*** 5: 387-392.
2. Zhu, K., J. Wang, J. Zhu, J. Jiang, J. Shou, and **X. Chen**. 1999. p53 activates the transporter associated with antigen processing 1 gene and enhances the transport of MHC I class I peptides. ***Oncogene*** 18: 7740-7747.
3. Wang, J., J. Shou, and **X. Chen**. 2000. Dickkopf-1, an inhibitor of the Wnt signaling pathway, is induced by p53. ***Oncogene*** 19: 1843-1848.
4. Zhu, J., S. Zhang, J. Jiang, and **X. Chen**. 2000. Definition of the p53 functional domains necessary for inducing apoptosis. ***Submitted to J. Biol. Chem.***
5. Abstract for the presentation at the American Association for Cancer Research 91th annual meeting, San Francisco, CA. April 1-5, 2000.
6. Abstract for presentation at the DOD Breast Cancer Research Program Meeting, Era of Hope, Atlanta, GA. June 8-11, 2000.
7. Abstract for presentation at the 10^{th} p53 workshop, Monterey, CA. April 5-8, 2000.

(5) Introduction

The p53 protein contains several functional domains necessary for inducing cell cycle arrest and apoptosis, that is, activation domain 1, within residues 1-42 (1-3), the proline-rich domain, within residues 64-91 (4), and the C-terminal basic domain, within residues 364-393 (5). Recently, we and others have shown that p53(AD1$^-$) contains an intact activation domain 2, within residues 43-63 (6-8), and therefore, p53(AD1$^-$) is still competent in transactivation (7). Furthermore, when both activation domain 1 and activation domain 2 are mutated (a quadruple point mutation at residues 22-23 and 53-54; AD1$^-$AD2$^-$), the resulting protein is inert in transactivation and in inducing cell cycle arrest and apoptosis (6-8). The proline-rich domain has been shown to be required for efficient growth suppression (4). We and others have shown that the proline-rich domain is necessary for apoptosis but not cell cycle arrest (9-11). In addition, the proline-rich domain plays an important role in the induction of several endogenous target genes, but is not required for activation of the exogenously introduced promoters of these target genes (10). These results suggest that the proline-rich domain may participate in the induction of cellular target gene(s) responsible for mediating apoptosis. The C-terminal basic domain has been subjected to extensive analysis and all evidence suggests that the basic domain is a negative regulatory domain. However, several groups have shown that p53(ΔBD), which lacks the C-terminal basic domain, has a reduced ability to induce several cellular target genes and becomes incapable of inducing apoptosis (12-14). These results suggest that the C-terminal basic domain can regulate p53 activity both positively and negatively.

(6) Body

To further determine the role of each p53 functional domain in inducing cell cycle arrest and apoptosis, we generated a number of H1299 and MCF7 cell lines that inducibly express p53 that lacks one or more of the domains. We found that an activation domain 2 mutation at residues 53-54 (AD2$^-$) abrogates the apoptotic activity but has no significant effect on cell cycle arrest. We also found that p53(ΔAD2), which lacks activation domain 2, are inert in inducing apoptosis. p53(AD2$^-$ΔBD), which is defective in activation domain 2 and lacks the C-terminal basic domain, p53(ΔAD2ΔBD), which lacks both activation domain 2 and the C-terminal basic domain, and p53(ΔPRDΔBD), which lacks both the proline-rich domain and the C-terminal basic domain, are also inert in inducing apoptosis. All four mutants are still active in inducing cell cycle arrest, albeit to a lesser extent than wild-type p53. Interestingly, we found that deletion of the N-terminal activation domain 1 alleviates the requirement of the C-terminal basic domain for apoptotic activity. Thus, we have generated a small but potent p53(ΔAD1ΔBD) molecule. Furthermore, we found that at least two of the three domains, that is, activation domain 1, activation domain 2 and the proline-rich domain, are required for inducing cell cycle arrest. Taken together, our results suggest that activation domain 2 and the proline-rich domain form an activation domain for inducing pro-apoptotic genes or inhibiting anti-apoptotic genes. The C-terminal basic domain is required for maintaining this activation domain competent for transactivation or transrepression. For detail, please see the attached manuscript, appendix #4.

As an extension of Tasks 8-10, we have identified two p53 target genes that may mediate p53 tumor suppression. Dickkopf-1 (Dkk-1), a secreted glycoprotein, has been found to be

necessary and sufficient for inducing amphibian head formation. Interestingly, the mechanism by which Dkk-1 does this is the ability of Dkk-1 to antagonize the Wnt signaling pathway. Wnt, itself a proto-oncoprotein, can promote cell proliferation and transformation when mutated or overexpressed, leading to tumor formation. p53 is a tumor suppressor and loss of p53 function accelerates mammary tumorigenesis by Wnt. Here we found that Dkk-1 is induced by wild-type p53 but not mutant p53(R249S). In addition, DNA damage upregulates Dkk-1 in cell lines that harbor an endogenous wild-type p53 gene but not in cell lines that are p53-null or harbor an endogenous mutant p53 gene. We also found a potential p53 response element located approximately 2,100 nucleotides upstream of the Dkk-1 transcription start site and we show that p53 binds specifically to this element both *in vitro* and *in vivo*. Furthermore, we have established several cell lines derived from H1299 lung carcinoma and U118 glioma cells that inducibly express Dkk-1 under a tetracycline-regulated promoter. We found that Dkk-1 has no effect on proliferation of cells that are not transformed by Wnt. Taken together, these results suggest that Dkk-1 may mediate p53 tumor suppression by antagonizing the Wnt signaling pathway. For detail, please see the attached manuscript, appendix #3.

The transporter associated with antigen processing (TAP) 1 is required for the major histocompatibility complex (MHC) class I antigen presentation pathway, which plays a key role in host tumor surveillance. Since more than 50% of tumors have a dysfunctional p53, evasion of tumor surveillance by tumor cells may be linked to loss of p53 function. Here we found that TAP1 is strongly induced by p53 and DNA-damaging agents through a p53 response element. We also found that p73, which is homologous to p53, is capable of inducing TAP1 and cooperates with p53 to activate TAP1. Furthermore, we found that by inducing TAP1, p53 enhances the transport of MHC class I peptides and expression of surface MHC-peptide complexes, and cooperates with interferon γ to activate the MHC class I pathway. These results suggest that tumor surveillance may be a mechanism by which p53 and/or p73 function as tumor suppressors. For detail, please see the attached manuscript, appendix #2.

Work accomplished in relation to the Statement of Work

Tasks 1-2: A number of MCF7 breast carcinoma cell lines that inducibly express p53 or p73 using a tetracycline-regulated promoter have been generated and analyzed (1998 and 1999 annual report). During the third year of this grant, we have generated a number of MCF7 and H1299 cell lines that express various mutated forms of p53 (2000 annual report).

Tasks 3: DNA damage enhances p73-dependent apoptosis in MCF7 but not in MCF7E6 cells (1999 annual report). We will determine how DNA damage affects p53-dependent apoptosis in MCF7 cells.

Tasks 4-5: We found that transient overexpression of cyclin D1 in MCF7 cells can enhance DNA damage-induced p53-dependent apoptosis. We also found that transient coexpression of p53 and cyclin D1 in MCF7 cells can induce a strong apoptotic response. Since similar results have been published by other group (15), we will spend more effort on tasks 8-10.

Task 6: A number of short deletion and point mutations of p53 have been generated and analyzed (1998, 1999, and 2000 annual report).

Task 7: We identified a novel apoptotic domain and activation domain II in p53 (1998 annual report). We showed that the proline-rich domain is necessary for apoptosis (1999 annual report). During the third year of this grant, we have generated a small but potent p53(ΔAD1ΔBD) molecule. Furthermore, we found that at least two of the three domains, that is, activation domain 1, activation domain 2 and the proline-rich domain, are required for inducing cell cycle arrest. Our results suggest that activation domain 2 and the proline-rich domain form an activation domain for inducing pro-apoptotic genes or inhibiting anti-apoptotic genes. The C-terminal basic domain is required for maintaining this activation domain competent for transactivation or transrepression.

Task 8-10: We showed that p73 functionally interacts with p53 in cells and activation of the p53 pathway is necessary for the cooperative induction of apoptosis between p73 and DNA damage in MCF7 cells (1999 annual report). We will perform additional experiments to further characterize both the functional and physical interactions between p53 and p73 and prepare a manuscript for publication. As an extension of these tasks, we have identified two p53 target genes, DKK1 and TAP1, which are capable of mediating p53 tumor suppression (2000 annual report).

(7) Key Research Accomplishments for the period of July 1, 1999 to June 30, 2000

- A small but potent p53(ΔAD1ΔBD) molecule was generated, which can induce a strong apoptotic response in MCF7 cells.
- We found that at least two of the three domains, that is, activation domain 1, activation domain 2 and the proline-rich domain, are required for inducing cell cycle arrest.
- Our results suggest that activation domain 2 and the proline-rich domain form an activation domain for inducing pro-apoptotic genes or inhibiting anti-apoptotic genes. The C-terminal basic domain is required for maintaining this activation domain competent for transactivation or transrepression.
- p53 induces the transporter associated with antigen processing 1 gene and enhances the transport of MHC I class I peptides, suggesting that p53 plays a role in immunosurveillance.
- p53 induces Dickkopf-1, an inhibitor of the Wnt signaling pathway, suggesting that Dickkopf-1 may mediate p53 tumor suppression by antagonizing the Wnt signaling pathway.

(8) Reportable Outcomes for the period of July 1, 1999 to June 30, 2000

1. **Chen, X.** 1999. The p53 family: same response, different signals? ***Molecular Medicine Today*** 5: 387-392.
2. Zhu, K., J. Wang, J. Zhu, J. Jiang, J. Shou, and **X. Chen**. 1999. p53 activates the transporter associated with antigen processing 1 gene and enhances the transport of MHC I class I peptides. ***Oncogene*** 18: 7740-7747.
3. Wang, J., J. Shou, and **X. Chen**. 2000. Dickkopf-1, an inhibitor of the Wnt signaling pathway, is induced by p53. ***Oncogene*** 19: 1843-1848.

4. Zhu, J., S. Zhang, J. Jiang, and **X. Chen**. 2000. Definition of the p53 functional domains necessary for inducing apoptosis. ***Submitted to J. Biol. Chem.***
5. A presentation at the American Association for Cancer Research 91th annual meeting, San Francisco, CA. April 1-5, 2000. Abstract #3958.
6. A presentation at the DOD Breast Cancer Research Program Meeting, Era of Hope, Atlanta, GA. June 8-11, 2000. Poster # B-77.
7. A presentation at the 10th p53 workshop, Monterey, CA. April 5-8, 2000. Poster #32.

(9) Conclusions

p53(ΔAD1ΔBD) lacks the MDM2 binding site (1) and would not be subjected to the negative regulation by MDM2. Thus, p53(ΔAD1ΔBD) represents a small but potent, apoptosis-inducing form of p53. Recent clinical tries have shown that adenoviruses expressing p53 are effective in treating some advanced forms of human cancers (16,17). We suggest that p53(ΔAD1ΔBD) is a good candidate to replace the larger, unwieldy wild-type p53 in cancer gene therapy.

The identification of TAP1 as a novel p53 and p73 target gene suggests that p53 and p73 function as tumor suppressors by regulating host immunosurveillance. Supporting this idea is the observation that deficiencies in p53 and p73 render mice susceptible to chronic infections, inflammation, and death due to unresolved infections. Therefore, this finding will lead us to further determine the role of immunosurveillance in p53 and p73 tumor suppression.

(10) References

1. Lin, J., Chen, J., Elenbaas, B., and Levine, A. J. (1994) *Genes Dev* **8**(10), 1235-46
2. Unger, T., Mietz, J. A., Scheffner, M., Yee, C. L., and Howley, P. M. (1993) *Mol Cell Biol* **13**(9), 5186-94
3. Chang, J., Kim, D. H., Lee, S. W., Choi, K. Y., and Sung, Y. C. (1995) *J Biol Chem* **270**(42), 25014-9
4. Walker, K. K., and Levine, A. J. (1996) *Proc Natl Acad Sci U S A* **93**(26), 15335-40
5. Ko, L. J., and Prives, C. (1996) *Genes Dev* **10**(9), 1054-72
6. Candau, R., Scolnick, D. M., Darpino, P., Ying, C. Y., Halazonetis, T. D., and Berger, S. L. (1997) *Oncogene* **15**(7), 807-16
7. Zhu, J., Zhou, W., Jiang, J., and Chen, X. (1998) *J Biol Chem* **273**(21), 13030-6
8. Venot, C., Maratrat, M., Sierra, V., Conseiller, E., and Debussche, L. (1999) *Oncogene* **18**(14), 2405-10
9. Sakamuro, D., Sabbatini, P., White, E., and Prendergast, G. C. (1997) *Oncogene* **15**(8), 887-98
10. Zhu, J., Jiang, J., Zhou, W., Zhu, K., and Chen, X. (1999) *Oncogene* **18**(12), 2149-55
11. Venot, C., Maratrat, M., Dureuil, C., Conseiller, E., Bracco, L., and Debussche, L. (1998) *Embo J* **17**(16), 4668-79
12. Chen, X., Ko, L. J., Jayaraman, L., and Prives, C. (1996) *Genes Dev* **10**(19), 2438-51

13. Wang, X. W., Vermeulen, W., Coursen, J. D., Gibson, M., Lupold, S. E., Forrester, K., Xu, G., Elmore, L., Yeh, H., Hoeijmakers, J. H., and Harris, C. C. (1996) *Genes Dev* **10**(10), 1219-32
14. Zhou, X., Wang, X. W., Xu, L., Hagiwara, K., Nagashima, M., Wolkowicz, R., Zurer, I., Rotter, V., and Harris, C. C. (1999) *Cancer Res* **59**(4), 843-8
15. Coco Martin, J. M., Balkenende, A., Verschoor, T., Lallemand, F., and Michalides, R. (1999) *Cancer Res* **59**(5), 1134-40
16. Swisher, S. G., Roth, J. A., Nemunaitis, J., Lawrence, D. D., Kemp, B. L., Carrasco, C. H., Connors, D. G., El-Naggar, A. K., Fossella, F., Glisson, B. S., Hong, W. K., Khuri, F. R., Kurie, J. M., Lee, J. J., Lee, J. S., Mack, M., Merritt, J. A., Nguyen, D. M., Nesbitt, J. C., Perez-Soler, R., Pisters, K. M., Putnam, J. B., Jr., Richli, W. R., Savin, M., Waugh, M. K., and et al. (1999) *J Natl Cancer Inst* **91**(9), 763-71
17. Clayman, G. L., el-Naggar, A. K., Lippman, S. M., Henderson, Y. C., Frederick, M., Merritt, J. A., Zumstein, L. A., Timmons, T. M., Liu, T. J., Ginsberg, L., Roth, J. A., Hong, W. K., Bruso, P., and Goepfert, H. (1998) *J Clin Oncol* **16**(6), 2221-32

The p53 family: same response, different signals?

Xinbin Chen

TP53, the gene that encodes p53, is a well-defined tumor suppressor gene that is frequently mutated in human cancers. Recently, two proteins homologous to p53, termed p73 and p63, were identified. Current data indicate that both p73 and p63, like p53, can induce cell-cycle arrest and apoptosis, suggesting that they might also be tumor suppressors. However, the physiological signals that can regulate p53, for example, DNA damage, have no effect on p73, as tested in several cell lines. Furthermore, the signaling pathways by which p73 (and possibly p63) induces cell-cycle arrest and apoptosis appear to be similar to those of p53, but also have important differences. Thus, the p53 family proteins are closely related but might have distinct physiological functions.

MOST tumor suppressor genes belong to families with several members. For almost two decades, no other *TP53* family member was identified, and it was believed to be an orphan without a family; but no longer. In fact, the *TP53* family has become quite large. Its first relative, *TP73*, which encodes p73, was identified by chance in 1997 (Ref. 1), and its second, *TRP63* (which encodes p63 and is also known as *KET*, *P51*, *P40*, chronic ulcerative stomatitis protein (CUSP) and *P73L*), was identified independently by several groups[2–7]. Furthermore, p53CP, a 40-kDa polypeptide[8], and NBP (non-p53 p53RE binding protein), a 44-kDa polypeptide[9], were also found to be capable of specifically binding to the same DNA element as does p53. Although the genes that encode p53CP and NBP have not yet been cloned, these proteins could be new members of the p53 family or alternatively spliced forms of the existing p53 family proteins.

Human p53 comprises 393 amino acid residues (Fig. 1). The *TP53* gene consists of 11 exons and is located at chromosome 17p13.1. Alternative splicing of intron 9 in human p53 mRNA leads to production of a protein truncated at the C-terminus (p53AS)[10]. It is well documented that mouse p53 and p53AS have different patterns of production and different activities[11,12] but the significance of human p53AS in tumor suppression remains to be elucidated. There are at least four alternatively spliced forms of human p73: p73α, p73β, p73γ and p73δ (Fig. 1)[1,13]. The *TP73* gene contains 14 exons and is located at chromosome 1p36.33 (Ref. 1). The human p63 subgroup of proteins contains the most variants within the p53 family[2–4]. The *TRP63* gene is located at chromosome 3q27–29 and contains 15 exons (Fig. 1)[2,4]. The *TRP63* gene can be transcribed from two different promoters, which are located upstream of exon 1 and within intron 3, respectively[2]. Alternative splicing of p63 mRNA transcribed from the upstream promoter leads to the production of three spliced forms of p63: p63α, p63β and p63γ (Ref. 2). When *TRP63* is transcribed from the **cryptic promoter** in intron 3, three N-terminal truncated proteins, ΔNp63α, ΔNp63β and ΔNp63γ, are produced[2]. A splicing variant that deletes four amino acids in exon 9 was detected in both the p63 and ΔNp63 species[2]. The human KET protein appears to be translated from the alternative start site of the p63α transcript, generating 39 extra residues at the N-terminus[5]. Because the C-terminal 114 amino acids in p40 are different from other p63 variants, p40 is one of the p63 isoforms[3]. Altogether, the *TRP63* gene encodes at least 14 variants of p63.

The p53 protein contains several functional domains (Fig. 2b; Ref. 14 and references therein): **activation domain** 1 (AD1) and AD2, located within residues 1–42 and 43–63, respectively[15,16]; five proline-rich **growth-suppression motifs**[17], located within residues 64–90; a sequence-specific DNA-binding domain, located within residues 100–300; a nuclear localization signal (NLS), located within residues 316–325; an oligomerization domain, located within residues 334–356; and a basic C-terminal regulatory domain, located within residues 363–393. A comparison of p53 sequences from various vertebrates revealed five evolutionarily conserved boxes. Box I is in AD1 (Refs 18,19) and boxes II to V are in the sequence-specific DNA-binding domain. Importantly, the vast majority of p53 missense mutations found in human tumors are clustered in the sequence-specific DNA-binding domain. Indeed, both p73 and p63 were identified as p53 family members because they contain a DNA-binding domain homologous to that of p53 (Refs 1–4).

How do p73 and p63 compare with p53? The homology between p53 and the other family members is extensive within the most conserved p53 functional domains (Fig. 2). Residues 1–59 in p63 and 1–54 in p73 are 22% and 29% identical, respectively, to residues 1–45 of AD1 in p53. Residues 142–321 in p63 and 131–310 in p73 are 60% and 63% identical, respectively, to residues 113–290 of the sequence-specific

Xinbin Chen PhD
Assistant Professor

Institute of Molecular Medicine and Genetics, CB-2803/IMMAG, Medical College of Georgia, Augusta, GA 30912, USA.
Tel: +1 706 721 8760
Fax: +1 706 721 8752
e-mail: xchen@mail.mcg.edu

1357-4310/99/$ - see front matter © 1999 Elsevier Science Ltd. All rights reserved. PII: S1357-4310(99)01545-2

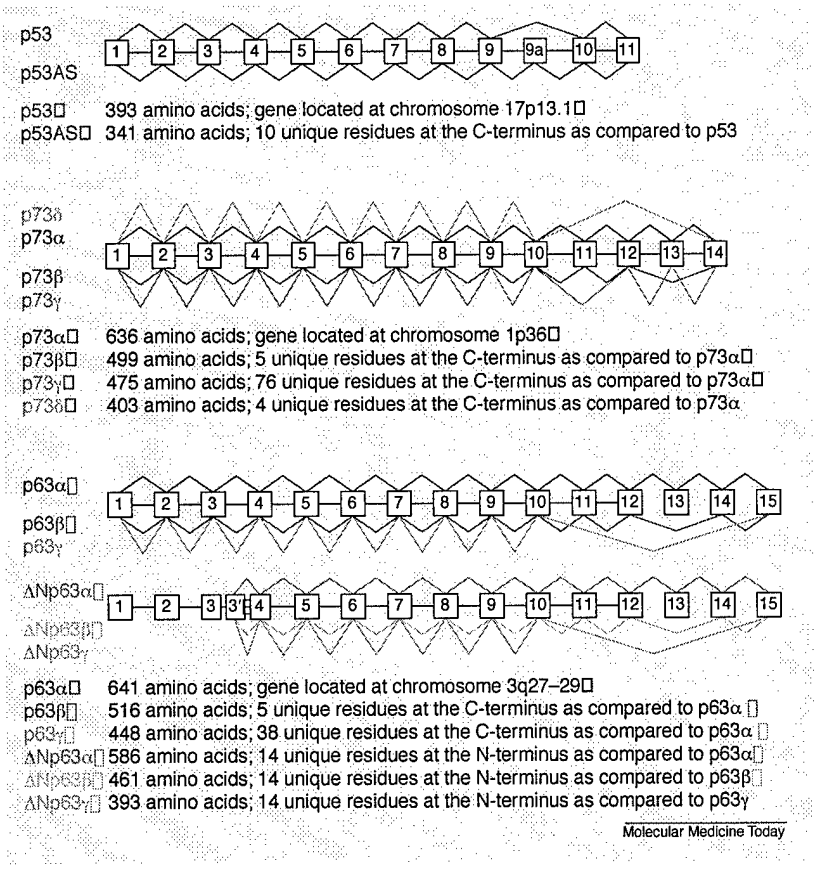


Figure 1. Gene organization of the P53 family members. Exons are shown as numbered boxes and introns as lines. TRP63 is also known as KET, P51, P40, CUSP and P73L. The p51B protein is identical to p63α, p51A identical to p63γ, p73L and CUSP identical to ΔNp63α, p40 identical to ΔNp63 except the C-terminal 114 amino acids, and KET identical to p63α except the N-terminal 39 amino acids. Modified from Refs 1,2,13.

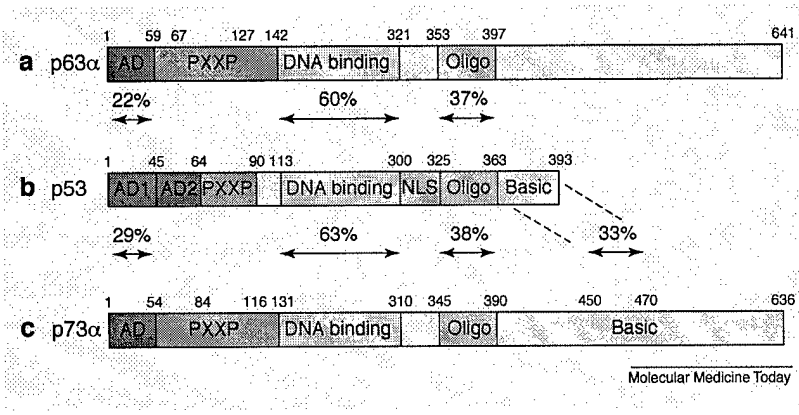


Figure 2. Homology among the p53 family of proteins, (**a**) p63α, (**b**) p53 and (**c**) p73α. Numbers above double-arrowed bars represent the percentage of p53-identical residues found in p63 or p73. Numbers above the bars are amino acid numbers. Abbreviations: AD, **activation domain**; Basic, p53 C-terminal basic domain; DNA binding, sequence-specific DNA-binding domain; NLS, nuclear localization signal; Oligo, oligomerization domain; **PXXP**, proline-rich domain where P represents proline and X any amino acid.

DNA-binding domain in p53. Residues 353–397 in p63 and 345–390 in p73 are 37% and 38% identical, respectively, to residues 319–363 of the oligomerization domain in p53. Although homology in the **PXXP motifs** between p53 and other p53 family members is not significant, both p63 and p73 do contain two **PXXP motifs**. Interestingly, residues 450–470 in p73 are 33% identical to the C-terminal basic regulatory domain in p53, while such a domain is not present in p63. It should be mentioned that p63 and p73 are more homologous to each other than to p53. Overall, p63 is 53% identical to p73. Specifically, the identity between p63 and p73 is 30% in the **activation domain**, 87% in the DNA-binding domain and 65% in the oligomerization domain[2–4]. Furthermore, human and murine p63 are 99% identical, with only eight substitutions, whereas human and mouse p53 are only 77% identical. Phylogenetic analysis of p53, p63 and p73 indicated that p63 is the most primitive and ancient member of the p53 family, suggesting that p63 might in fact be the evolutionary ancestor of the p53 family[2].

Are *TP73* and *TRP63* tumor suppressor genes?

TP53 is a bona fide tumor suppressor gene because it fulfills the 'classical features' of tumor suppressors (Table 1)[20], namely: (1) loss of function mutations accompanied by loss of heterozygosity occur in tumors; (2) in Li–Fraumeni syndrome, which predisposes individuals to multiple early-onset cancers, one allele of *TP53* is constitutively mutated; (3) *TP53* mutations occur in ~50% of spontaneous human tumors; (4) overexpression of *TP53* inhibits the growth of transformed cells; and (5) p53-deficient mice develop tumors at an early age.

TP73 was initially classified as a possible tumor suppressor gene because it is related to *TP53*, it maps to chromosome 1p36.33, a region frequently deleted in neuroblastoma and other human cancers and it has been found to be monoallelically expressed owing to **genomic imprinting** (Table 1)[1]. Thus, hemizygous deletion of the 'expressable' allele would result in total loss of *TP73* expression in cells. However, the status of *TP73* as a tumor suppressor gene has been challenged by recent observations[21–26]. Notably, *TP73* can be biallelically expressed in both normal and tumor tissues or cell lines, including neuroblastoma; and mutation of the *TP73* gene occurs infrequently in human cancers[27,28]. However, not all tumor suppressor genes fulfill the classical features mentioned above. Among these is *CDKN2D*, which encodes $p19^{ARF}$ and is an

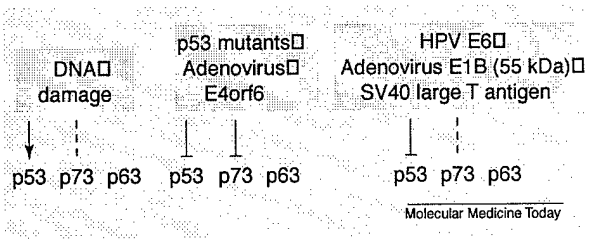


Figure 3. Modulation of the p53 family members by DNA damage, p53 mutants and viral oncoproteins. Arrow indicates activation. Blocked line indicates inhibition. Dashed line indicates no effect. Absence of line indicates not examined. It should be mentioned that although p73 is not induced by DNA damage and ultraviolet radiation in several neuroblastoma and non-neuroblastoma cell lines[1], it is still possible that some forms of DNA damage might affect p73 activity in certain tissues. In addition, more studies are needed to clarify the contradictory effect of the adenoviral E4orf6 oncoprotein on p73 (Refs 41–43) and to determine whether TP53 mutants other than TP53(R175H) and TP53(R248H) can inhibit p73 activity. Abbreviation: **HPV, human papillomavirus**.

alternatively spliced form of the *CDKN2A* tumor suppressor gene, which encodes $p16^{INK4a}$ (Ref. 29). Although homozygous deletions of *CDKN2D* occur in a wide range of human tumors, inactivating point mutations have not been found in the unique exon 1β of *CDKN2D*, which encodes the N-terminal 64 amino acids necessary for and sufficient to induce cell-cycle arrest[30,31]. Moreover, many inactivating point mutations found in *CDKN2A* are also predicted to alter *CDKN2D*, but those that have been tested experimentally do not affect the ability of $p19^{ARF}$ to induce cell-cycle arrest[30,31]. Interestingly, the mechanism by which *CDKN2D* functions as a tumor suppressor gene is its ability to regulate p53 function. Because *TP53* is frequently mutated, there might be no selective pressure for a mutated *CDKN2D*. In addition, p73 function can be inhibited by two tumor-derived *TP53* mutants, *TP53*(R175H) and *TP53*(R248H), in mammalian cells (Fig. 3)[32]. Therefore, in a similar manner, tumor cells with a mutated *TP53* gene would also have no selective pressure to mutate the *TP73* gene. Nevertheless, more studies are needed to determine whether *TP73* is a true tumor suppressor gene.

It is also not certain whether *TRP63* is a tumor suppressor gene (Table 1). *TRP63* is located at chromosome 3q27–29, a region that is not a common site of loss of heterozygosity in human cancers. The *TRP63* gene was found to be mutated, albeit infrequently, in both human tumor tissues and cancer cell lines[4]. Further complicating this matter is the observation that although p63 might have functions similar to those of p53 in cell-cycle arrest and apoptosis, ΔNp63, which lacks an **activation domain**, inhibits the activity of both p53 and p63, thereby exhibiting oncogenic functions[2]. Interestingly, *TRP63* is highly expressed in the basal region of many epithelial tissues[2] and is essential for limb, craniofacial and epidermal morphogenesis[33,34]. Therefore, to determine whether p63 plays any role in tumorigenesis will require extensive genetic and biochemical analyses.

Modulation of the p53 family proteins

Genomic instability is central to the development of cancer, and p53, by regulating the normal cellular response to DNA damage and other cellular insults, plays an essential role in the control of growth and division, thereby serving as a 'guardian of the genome'[35]. It is well documented that upon DNA damage, or under conditions of hypoxia or other cellular stresses, the p53 protein is stabilized (Fig. 3) and accumulation of the p53 protein leads to activation of checkpoint-control responses (for comprehensive reviews on this topic, see Refs 14,36–38). Although it is still not certain how p53 is stabilized, one mechanism for such a process is that p53 can be phosphorylated by a DNA-damage-inducible kinase, **ataxia telangiectasia-mutated (ATM)** kinase[39], and the phosphorylated p53 is then resistant to ubiquitin-dependent proteolysis. In contrast, p73 is not induced in several cell lines when treated with DNA-damaging agents, actinomycin D and doxorubicin, as well as ultraviolet and ionizing radiation[1]. The response of p63 to DNA damage remains to be determined.

p53 was originally identified as a protein that binds to the SV40 virus large T antigen[14,37]. It is believed that the physical interaction with and inactivation of p53 by viral oncoproteins, such as the SV40 large T antigen, adenovirus E1B 55-kDa protein and **human papillomavirus (HPV)** E6 protein, plays a central role in viral tumorigenesis[14,37]. However, recent experiments failed to show a physical interaction of p73 with the adenovirus E1B 55-kDa protein, **HPV** E6 protein and SV40 large T antigen both *in vitro* and *in vivo* (Fig. 3)[40–43]. Thus, these viral oncoproteins do not inhibit p73 function and the stable binding of these viral oncoproteins to p73 is apparently not necessary for transformation. The E1B 55-kDa protein can associate with p53, but not with p73, owing to the presence of an E1B 55-kDa-binding domain in p53, which is not present in p73 (Ref. 42). In domain-swapping experiments, five residues present in p53 (24-KLLPE-28), but not in the equivalent positions in p73 (20-SSLEP-24), were found to be necessary for E1B 55-kDa protein binding. However, one viral oncoprotein, adenovirus

Table 1. Members of the p53 family: how do they weigh up as tumor suppressors?

Characteristic[a]	p53	p63	p73
Loss-of-function mutations and LOH	Yes	Not found	Imprinted?[b]
Mutations in inherited cancer syndromes	Li–Fraumeni[18]	Not found	Not found
Somatic mutations in spontaneous tumors	Yes	Rare[c]	Rare[d]
Growth inhibition (cell-cycle arrest and apoptosis)	Yes	Yes[e]	Yes
Phenotype of mutant mice	Developmentally normal[64]; susceptible to spontaneous tumors	Postnatal lethal[33,34]; limb, craniofacial and epidermal morphogenesis	Not done

[a]The characteristics that define a tumor suppressor gene are taken from Ref. 20.
[b]p73 was found to be expressed monoallelically[1] and biallelically[21–25]. Loss of heterozygosity (LOH) for p73 occurs frequently in neuroblastomas[1,27].
[c]One somatic p63 missense mutation found in 66 human primary tumors[4].
[d]Mutations in p73 occur but infrequently[27,28].
[e]p63 but not ΔNp63 can induce cell-cycle arrest and apoptosis[2].

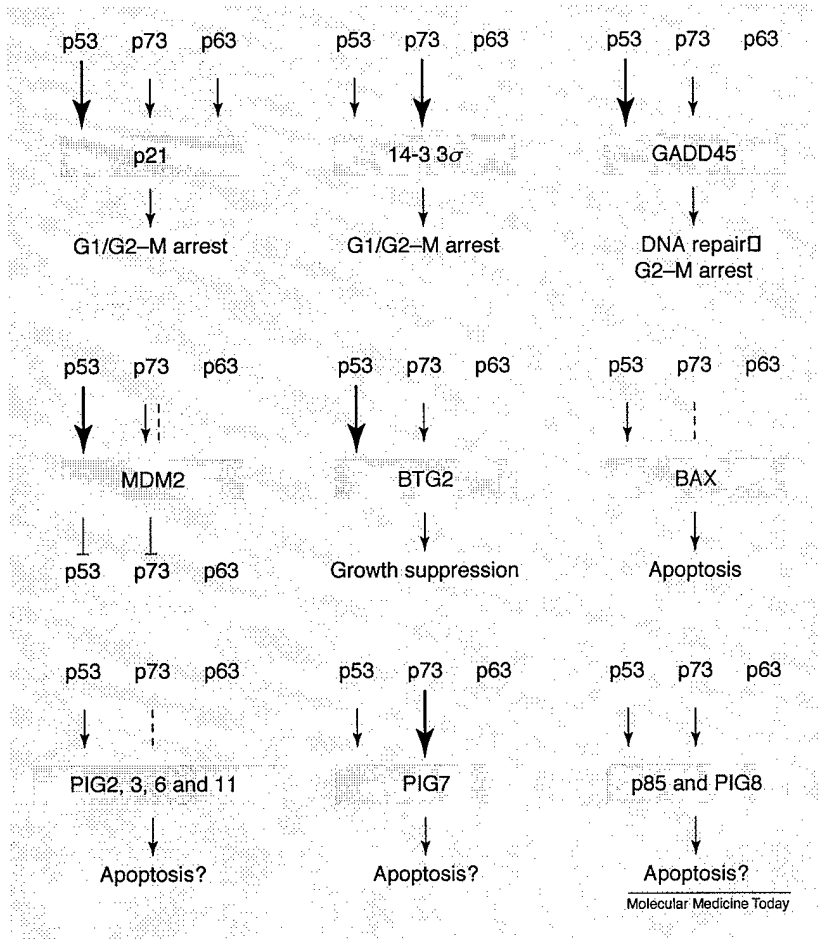


Figure 4. Regulation of the cellular target genes by the p53 family proteins. Heavy arrow indicates strong activation. Light arrow indicates weak activation. Blocked line indicates inhibition. Dashed line indicates no effect. Absence of line indicates not examined. Data obtained from Refs 1,2,4,44,45,62,63. Abbreviations: BTG2, B-cell translocation gene 2 antiproliferative; MDM2, human homolog of the murine double minute 2 gene; PIG, p53-induced gene.

Glossary

Activation domain – A region of a transcription factor that is required for its function. It might directly or indirectly interact with the basal transcription machinery, facilitating its assembly.

Ataxia telangiectasia-mutated (ATM) gene – A gene mutated in the autosomal recessive disorder ataxia telangiectasia (AT). The gene product is a member of the phosphoinositide 3-kinase (PI 3-kinase) family.

Cryptic promoter – Also called an alternate promoter; a DNA sequence that can control RNA transcription in certain tissues or in response to certain physiological stimuli.

Genomic imprinting – A phenomenon whereby a gene on the paternally and maternally derived chromosomes is differentially expressed.

Growth-suppression motif – A protein domain that is necessary for inhibiting cell proliferation; for example, the PXXP motif in p53.

Human papillomavirus (HPV) E6 – An HPV-encoded oncoprotein that can bind to p53 and facilitate ubiquitin ligation to p53, leading to degradation of p53.

PXXP motif – A motif that can bind to SH3 (Src homology 3) domains; P represents proline and X any amino acid.

E4orf6, was capable of associating with p73 and inhibiting the activity of p73 in one experimental protocol[41,43], but not in another[42]. Thus, further studies are needed to address this issue. Whether these viral oncoproteins can regulate p63 remains to be determined.

The signaling pathways of the p53 family proteins leading to tumor suppression

Like p53, p73 and p63 can induce cell-cycle arrest and apoptosis[1,2,4,44,45], both of which constitute the basic cellular mechanisms by which p53 mediates tumor suppression; and like p53, p73 and p63 are transcription factors[1,2,4,44]. Loss of p73 transcriptional activity abrogates its activity in cell-cycle arrest and apoptosis[1,44,45]. It is well known that p53 upregulates p21, an inhibitor of the cyclin-dependent kinases[46–48]. This is primarily responsible for p53-dependent arrest in G1 phase. Consistent with their ability to induce cell-cycle arrest, both p73 and p63 can induce p21 synthesis (Fig. 4)[1,4,44,45]. Although p73 can induce p21, the level of cellular p21 induced by p73 is several times lower than that induced by p53 (Ref. 45). Two other p53 target genes that can cause growth suppression and might be involved in cell-cycle arrest, *GADD45* (Ref. 49) and B-cell translocation gene 2 antiproliferative (*BTG2*; Ref. 50), are only weakly activated by p73 (Fig. 4)[45]. Therefore, it remains to be determined whether the level of p21 induced by p73 is sufficient to cause cell-cycle arrest and whether other cellular genes might also be involved in p73-dependent cell-cycle arrest.

p53-dependent G2–M arrest is mediated, at least in part, by upregulation of a gene known as *14-3-3σ* (Ref. 51). The product of this gene interacts with the cdc25 phosphatase to block activation of the cyclin B-dependent cdc2 kinase, which is required for initiation of mitosis[52]. Consistent with the observation that p73 can induce G2–M arrest, p73 is capable of inducing *14-3-3σ* (Fig. 4)[45]. Interestingly, p73 induces several-fold higher levels of the *14-3-3σ* gene product than does p53. These results suggest that a signaling pathway to induce arrest in G2–M is conserved between p53 and p73, and that *14-3-3σ* might be a *bona fide* cellular target gene of p73, even though it was originally identified as a potential p53 target gene. It remains to be determined whether p63 can induce *14-3-3σσ* and cell-cycle arrest in G2–M.

The outstanding questions

- What are the physiological signals that regulate p63 and/or p73? Do the signals that regulate p53, such as DNA damage, hypoxia and nucleotide deprivation, also regulate p63 and p73?
- Are there cellular target genes that are regulated specifically by p63 or p73? What are the common targets among the p53 family members?
- What are the domains in p63 and p73 necessary for growth suppression? Are the domains in p63 and p73 separable for cell-cycle arrest and apoptosis?
- Do functional interactions exist among p53, p63 and p73 in cells under physiological conditions?
- Is there a possibility that activating p63 and p73 might be a useful therapeutic strategy for tumors that have lost p53 activity?

Although both p53 and p73 can induce apoptosis[44,45], the signaling pathways used might differ, based on the differential ability of p73 to activate some p53 target genes. *BAX* and several redox-related genes [p53-induced gene 2 (*PIG2*), *PIG3*, *PIG6* and *PIG11*] that might be involved in mediating p53-dependent apoptosis[53,54] were not significantly induced by p73 (Fig. 4)[45]. Although *PIG7*, *PIG8* and *P85* were induced by P73 (Fig. 4)[45], the functions of *PIG7* and *PIG8* in apoptosis are still unknown and the role of *P85* in apoptosis appears to be restricted to the cellular response to oxidative stress[55]. Because p73 transcriptional activity is required for inducing apoptosis[1,44,45], it is possible that a distinctive group of cellular genes that can be activated by p73 might be responsible for mediating apoptosis. The signaling pathway for p63 induction of apoptosis remains to be determined.

The human homolog of the murine double minute 2 gene (*MDM2*), an oncogene that negatively regulates p53 and is also induced by p53 (Ref. 56), is weakly induced by p73β but not by p73α (Fig. 4)[45]. MDM2 binds to p53, enhancing the degradation of p53 through the ubiquitination pathway[57–60], as well as concealing the **activation domain** of p53 (Ref. 61), thus abolishing its ability to regulate transcription. Interestingly, MDM2 binds to and suppresses p73 function without promoting p73 degradation[62,63]. The domain required for MDM2 binding is present in the **activation domain** of p63 (Ref. 2), but whether p63 can regulate *MDM2* or be regulated by MDM2 remains to be elucidated.

Prospects for the future

Among the most pressing issues is the identification of physiological signals that regulate p63 and/or p73 activity. In addition, dissecting the domain(s) necessary for p63- and p73-dependent cell-cycle arrest and apoptosis might also provide insights into how p63 and p73 are regulated by physiological conditions and might be informative for engineering a more potent p63 or p73 for use as a therapeutic agent. Because p63 and p73 are infrequently mutated in human cancers, activating p63 and/or p73 pathways in cells that have lost p53 activity might be a useful therapeutic strategy. Furthermore, it will be interesting to determine whether and how p63 or p73 cooperate with p53 to mediate tumor suppression; this will guide future decisions as to whether p63 or p73 should potentially be used with p53 for gene therapy.

Acknowledgements. I thank R. Markowitz, L. Ko and S. Nozell for critically reading this manuscript. The work is supported in part by National Cancer Institute Grant CA81237 and the United States Department of Defense Army Breast Cancer Program Grant DAMD17-97-I-7019.

References

1 Kaghad, M. *et al.* (1997) **Monoallelically expressed gene related to *p53* at 1p36, a region frequently deleted in neuroblastoma and other human cancers,** *Cell* 90, 809–819

2 Yang, A. *et al.* (1998) ***p63*, a *p53* homolog at 3q27–29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities,** *Mol. Cell* 2, 305–316

3 Trink, B. *et al.* (1998) **A new human p53 homologue,** *Nat. Med.* 4, 747–748

4 Osada, M. *et al.* (1998) **Cloning and functional analysis of human p51, which structurally and functionally resembles p53,** *Nat. Med.* 4, 839–843

5 Augustin, M., Bamberger, C., Paul, D. and Schmale, H. (1998) **Cloning and chromosomal mapping of the human p53-related *KET* gene to chromosome 3q27 and its murine homolog *Ket* to mouse chromosome 16,** *Mamm. Genome* 9, 899–902

6 Senoo, M. *et al.* (1998) **A second p53-related protein, p73L, with high homology to *p73*,** *Biochem. Biophys. Res. Commun.* 248, 603–607

7 Lee, L.A. *et al.* **Characterization of an autoantigen associated with chronic ulcerative stomatitis: the CUSP autoantigen is a member of the p53 family,** *J. Invest. Dermatol.* (in press)

8 Bian, J. and Sun, Y. (1997) **p53CP, a putative p53 competing protein that specifically binds to the consensus p53 DNA binding sites: a third member of the p53 family?** *Proc. Natl. Acad. Sci. U. S. A.* 94, 14753–14758

9 Zeng X., Levine, A.J. and Lu, H. (1998) **Non-p53 p53RE binding protein, a human transcription factor functionally analogous to p53,** *Proc. Natl. Acad. Sci. U. S. A.* 95, 6681–6686

10 Flaman, J.M. *et al.* (1996) **The human tumour suppressor gene p53 is alternatively spliced in normal cells,** *Oncogene* 12, 813–818

11 Rehberger, P.A. *et al.* (1997) **Differential expression of the regularly spliced wild-type p53 and its COOH-terminal alternatively spliced form during epidermal differentiation,** *Cell Growth Differ.* 8, 851–860

12 Almog, N. *et al.* (1997) **The murine C′-terminally alternatively spliced form of p53 induces attenuated apoptosis in myeloid cells,** *Mol. Cell. Biol.* 17, 713–722

13 De Laurenzi, V. *et al.* (1998) **Two new p73 splice variants, gamma and delta, with different transcriptional activity,** *J. Exp. Med.* 188, 1763–1768

14 Ko, L.J. and Prives, C. (1996) **p53: puzzle and paradigm,** *Genes Dev.* 10, 1054–1072

15 Zhu, J., Zhou, W., Jiang, J. and Chen, X. (1998) **Identification of a novel p53 functional domain that is necessary for mediating apoptosis,** *J. Biol. Chem.* 273, 13030–13036

16 Candau, R. *et al.* (1997) **Two tandem and independent sub-activation domains in the amino terminus of p53 require the adaptor complex for activity,** *Oncogene* 15, 807–816

17 Walker, K.K. and Levine, A.J. (1996) **Identification of a novel p53 functional domain that is necessary for efficient growth suppression,** *Proc. Natl. Acad. Sci. U. S. A.* 93, 15335–15340

18 Evans, S.C. and Lozano, G. (1997) **The Li–Fraumeni syndrome: an inherited susceptibility to cancer,** *Mol. Med. Today* 3, 390–395

19 Lin, J., Chen, J., Elenbaas, B. and Levine, A.J. (1994) **Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein,** *Genes Dev.* 8, 1235–1246

20 Clurman, B. and Groudine, M. (1997) **Tumour-suppressor genes. Killer in search of a motive?** *Nature* 389, 122–123

21 Nomoto, S. *et al.* (1998) **Search for mutations and examination of allelic expression imbalance of the p73 gene at 1p36.33 in human lung cancers,** *Cancer Res.* 58, 1380–1383

22 Takahashi, H. *et al.* (1998) **Mutation, allelotyping, and transcription analyses of the *p73* gene in prostatic carcinoma,** *Cancer Res.* 58, 2076–2077

23 Kovalev, S. *et al.* (1998) **Expression level, allelic origin, and mutation analysis of the *p73* gene in neuroblastoma tumors and cell lines,** *Cell Growth Differ.* 9, 897–903

24 Tsao, H., Zhang, X., Majewski, P. and Haluska, F.G. (1999) **Mutational and expres-**

sion analysis of the *p73* gene in melanoma cell lines, *Cancer Res.* 59, 172–174

25 Mai, M. *et al.* (1998) **Activation of *p73* silent allele in lung cancer**, *Cancer Res.* 58, 2347–2349

26 Mai, M. *et al.* (1998) **Loss of imprinting and allele switching of *p73* in renal cell carcinoma**, *Oncogene* 17, 1739–1741

27 Ichimiya, S. *et al.* (1999) ***p73* at chromosome 1p36.3 is lost in advanced stage neuroblastoma but its mutation is infrequent**, *Oncogene* 18, 1061–1066

28 Han, S. *et al.* (1999) **Infrequent somatic mutations of the *p73* gene in various human cancers**, *Eur. J. Surg. Oncol.* 25, 194–198

29 Sherr, C.J. (1998) **Tumor surveillance via the ARF–p53 pathway**, *Genes Dev.* 12, 2984–2991

30 Zhang, Y., Xiong, Y. and Yarbrough, W.G. (1998) **ARF promotes MDM2 degradation and stabilizes p53: *ARF-INK4a* locus deletion impairs both the Rb and p53 tumor suppression pathways**, *Cell* 92, 725–734

31 Quelle, D.E., Cheng, M., Ashmun, R.A. and Sherr, C.J. (1997) **Cancer-associated mutations at the INK4a locus cancel cell cycle arrest by p16INK4a but not by the alternative reading frame protein p19ARF**, *Proc. Natl. Acad. Sci. U. S. A.* 94, 669–673

32 Di Como, C.J., Gaiddon, C. and Prives, C. (1999) **p73 function is inhibited by tumor-derived p53 mutants in mammalian cells**, *Mol. Cell. Biol.* 19, 1438–1449

33 Mills, A.A. *et al.* (1999) **p63 is a p53 homologue required for limb and epidermal morphogenesis**, *Nature* 398, 708–713

34 Yang, A. *et al.* (1999) **p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development**, *Nature* 398, 714–718

35 Lane, D.P. (1992) **p53, guardian of the genome**, *Nature* 358, 15–16

36 Giaccia, A.J. and Kastan, M.B. (1998) **The complexity of p53 modulation: emerging patterns from divergent signals**, *Genes Dev.* 12, 2973–2983

37 Levine, A.J. (1997) **p53, the cellular gatekeeper for growth and division**, *Cell* 88, 323–331

38 Agarwal, M.L. *et al.* (1998) **The p53 network**, *J. Biol. Chem.* 273, 1–4

39 Canman, C.E. *et al.* (1998) **Activation of the ATM kinase by ionizing radiation and phosphorylation of p53**, *Science* 281, 1677–1679

40 Marin, M.C. *et al.* (1998) **Viral oncoproteins discriminate between p53 and the p53 homolog p73**, *Mol. Cell. Biol.* 18, 6316–6324

41 Higashino, F., Pipas, J.M. and Shenk, T. (1998) **Adenovirus E4orf6 oncoprotein modulates the function of the p53-related protein, p73**, *Proc. Natl. Acad. Sci. U. S. A.* 95, 15683–15687

42 Roth, J. *et al.* (1998) **Inactivation of p53 but not p73 by adenovirus type 5 E1B 55-kilodalton and E4 34-kilodalton oncoproteins**, *J. Virol.* 72, 8510–8516

43 Steegenga, W.T. *et al.* (1999) **Distinct regulation of p53 and p73 activity by adenovirus EIA, E1B, and e4orf6 proteins**, *Mol. Cell. Biol.* 19, 3885–3894

44 Jost, C.A., Marin, M.C. and Kaelin, W.G., Jr (1997) **p73 is a human p53-related protein that can induce apoptosis**, *Nature* 389, 191–194

45 Zhu, J., Jiang, J., Zhou, W. and Chen, X. (1998) **The potential tumor suppressor p73 differentially regulates cellular p53 target genes**, *Cancer Res.* 58, 5061–5065

46 el-Deiry, W.S. *et al.* (1993) **WAF1, a potential mediator of p53 tumor suppression**, *Cell* 75, 817–825

47 Xiong, Y. *et al.* (1993) **p21 is a universal inhibitor of cyclin kinases**, *Nature* 366, 701–704

48 Harper, J.W. *et al.* (1993) **The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases**, *Cell* 75, 805–816

49 Wang, X.W. *et al.* (1999) **GADD45 induction of a G2/M cell cycle checkpoint**, *Proc. Natl. Acad. Sci. U. S. A.* 96, 3706–3711

50 Rouault, J.P. *et al.* (1996) **Identification of BTG2, an antiproliferative p53-dependent component of the DNA damage cellular response pathway**, *Nat. Genet.* 14, 482–486

51 Hermeking, H. *et al.* (1997) **14-3-3 sigma is a p53-regulated inhibitor of G2/M progression**, *Mol. Cell* 1, 3–11

52 Peng, C.Y. *et al.* (1997) **Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216**, *Science* 277, 1501–1505

53 Miyashita, T. *et al.* (1994) **Tumor suppressor p53 is a regulator of *bcl-2* and *bax* gene expression *in vitro* and *in vivo***, *Oncogene* 9, 1799–1805

54 Polyak, K. *et al.* (1997) **A model for p53-induced apoptosis**, *Nature* 389, 300–305

55 Yin, Y. *et al.* (1998) **Involvement of p85 in p53-dependent apoptotic response to oxidative stress**, *Nature* 391, 707–710

56 Wu, X., Bayle, J.H., Olson, D. and Levine, A.J. (1993) **The p53–mdm-2 autoregulatory feedback loop**, *Genes Dev.* 7, 1126–1132

57 Grossman, S.R. *et al.* (1998) **p300/MDM2 complexes participate in MDM2-mediated p53 degradation**, *Mol. Cell* 2, 405–415

58 Honda, R., Tanaka, H. and Yasuda, H. (1997) **Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53**, *FEBS Lett.* 420, 25–27

59 Kubbutat, M.H., Jones, S.N. and Vousden, K.H. (1997) **Regulation of p53 stability by Mdm2**, *Nature* 387, 299–303

60 Haupt, Y., Maya, R., Kazaz, A. and Oren, M. (1997) **Mdm2 promotes the rapid degradation of p53**, *Nature* 387, 296–299

61 Oliner, J.D. *et al.* (1993) **Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53**, *Nature* 362, 857–860

62 Zeng, X. *et al.* (1999) **MDM2 suppresses p73 function without promoting p73 degradation**, *Mol. Cell. Biol.* 19, 3257–3266

63 Dobbelstein, M., Wienzek, S., Konig, C. and Roth, J. (1999) **Inactivation of the p53 homologue p73 by the mdm2-oncoprotein**, *Oncogene* 18, 2101–2106

64 Donehower, L.A. *et al.* (1992) **Mice deficient for p53 are developmentally normal**

Late-breaking news

Since acceptance of this manuscript, the signals that can regulate and modify p73 have been unraveled. p73 can be stabilized by DNA damage in a c-Abl-dependent manner when cells are treated with cisplatin[1] and phosphorylated at a tyrosine residue by c-Abl when cells are gamma-irradiated[2,3]. However, it is not clear why p73 is neither phosphorylated when cells are treated with cisplatin nor stabilized when cells are irradiated with ultraviolet light[1]. Nevertheless, c-Abl directly transduces the DNA damage signals to p73 through its Src homology 3 domain, which interacts with the C-terminal PXXP motif of p73 (Refs 2,3). Both stabilization and tyrosine phosphorylation of p73 by c-Abl enhance the transcriptional and pro-apoptotic activity of p73 (Refs 1–3). Therefore, future studies should address whether other physiological signals that induce p53, such as hypoxia and nucleotide deprivation, can also induce p73.

References

1 Gong, J. *et al.* (1999) **The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage**, *Nature* 399, 806–809

2 Agami, R. *et al.* (1999) **Interaction of c-Abl and p73a and their collaboration to induce apoptosis**, *Nature* 399, 809–813

3 Yuan, Z-M. *et al.* (1999) **p73 is regulated by tyrosine kinase c-Abl in the apoptotic response to DNA damage**, *Nature* 399, 814–817

Oncogene (1999) 18, 7740–7747
© 1999 Stockton Press All rights reserved 0950–9232/99 $15.00
http://www.stockton-press.co.uk/onc

p53 induces TAP1 and enhances the transport of MHC class I peptides

Kuichun Zhu[1,2], Jian Wang[1,2], Jianhui Zhu[1,2], Jieyuan Jiang[1], Jiang Shou[1] and Xinbin Chen*[,1]

[1]*Institute of Molecular Medicine and Genetics, Medical College of Georgia, Augusta, Georgia, GA 30912, USA*

The transporter associated with antigen processing (TAP) 1 is required for the major histocompatibility complex (MHC) class I antigen presentation pathway, which plays a key role in host tumor surveillance. Since more than 50% of tumors have a dysfunctional p53, evasion of tumor surveillance by tumor cells may be linked to loss of p53 function. Here we found that TAP1 is strongly induced by p53 and DNA-damaging agents through a p53-responsive element. We also found that p73, which is homologous to p53, is capable of inducing TAP1 and cooperates with p53 to activate TAP1. Furthermore, we found that by inducing TAP1, p53 enhances the transport of MHC class I peptides and expression of surface MHC-peptide complexes, and cooperates with interferon γ to activate the MHC class I pathway. These results suggest that tumor surveillance may be a mechanism by which p53 and/or p73 function as tumor suppressors.

Keywords: p53; TAP1; MHC class I; interferon γ; tumor surveillance

Introduction

p53 is one of the most frequently mutated genes in cancer. More than 50% of all human tumors contain a dysfunctional p53 (Hollstein *et al.*, 1991). It is well established that p53 plays an important role in the regulation of cell cycle, apoptosis, differentiation, and in the maintenance of genome integrity (Chen, 1999; Almog and Rotter, 1998; Ko and Prives, 1996; Levine, 1997), all of which contribute to p53 tumor suppression. As a sequence-specific transcription factor, p53 up-regulates expression of several cellular genes, for example, p21 and 14-3-3σ that mediate p53-dependent cell cycle arrest (el-Deiry *et al.*, 1993; Hermeking, 1997), and BAX and a group of redox-related genes (PIGs) that may mediate p53-dependent apoptosis (Miyashita *et al.*, 1994; Polyak *et al.*, 1997).

p53 is a multifunctional protein. Mechanisms other than cell cycle arrest and apoptosis may also be involved in p53 tumor suppression. When normal cells become malignant, cellular proteins that are normally present at low levels may become over-expressed or the genes that encode these cellular proteins may become mutated, resulting in the production of tumor antigens (Old and Chen, 1998). These tumor antigens would then be processed and presented by the host major histocompatibility complex (MHC) class I antigen presentation pathway on the cell surface. Several proteins are necessary for the MHC class I pathway, including large multifunctional proteasome subunits 2 and 7 (LMP2 and LMP7), transporters associated with antigen processing 1 and 2 (TAP1 and TAP2), and two polypeptides for the MHC class I molecule, heavy chain HLA-ABC and light chain β_2 microglobulin (β_2M) (Pamer and Cresswell, 1998). LMP2 and LMP7 are involved in breaking down intracellular proteins into antigenic peptides. TAP1 and TAP2 are involved in the transport of these antigenic peptides from cytosol to endoplasmic reticulum where they bind to the assembled MHC class I molecules. The MHC-peptide complex is then transported to and expressed on the cell surface. Cytotoxic T lymphocytes (CTLs) recognize and attack cells with tumor antigens on the cell surface via an interaction between the T cell receptor and the MHC-peptide complex. However, during tumorigenesis, tumor cells acquire mutations that help them evade recognition by the immune system. One mechanism that tumor cells could use is to down-regulate the MHC class I pathway (Pamer and Cresswell, 1998; Restifo *et al.*, 1993b). Without stable MHC-peptide complexes on the cell surfaces, tumor cells evade CTL recognition.

As part of our ongoing effort to understand p53 function in cells, we used the ClonTech PCR-Select cDNA Subtraction assay to identify novel cellular p53 target genes. We found that TAP1 is specifically induced by both p53 and p73, which leads to enhanced transport of MHC class I peptides. These findings suggest that tumor surveillance can be mediated by the p53 family tumor suppressor proteins.

Results

Upregulation of TAP1 by p53

In an effort to identify new p53 target genes, the ClonTech PCR-Select cDNA Subtraction assay was performed using mRNA isolated from p53-3, a derivative of H1299 cell line that inducibly expresses p53 under a tetracycline-regulated promoter (Chen *et al.*, 1996b). Several cDNA fragments that may represent genes activated by p53 were isolated. After DNA sequencing, one subtracted cDNA fragment was found to be derived from the TAP1 gene. To confirm that TAP1 can be induced by p53, Northern blot analysis was performed using TAP1 cDNA as probe. We found that TAP1 was induced in p53-3 cells when p53 was expressed (Figure 1a, compare lanes 1 and 2). As a control, we tested expression of p21, a well-defined cellular p53 target gene (el-Deiry *et al.*, 1993).

*Correspondence: X Chen
[2]These authors contributed equally to the study
Received 24 May 1999; revised 9 September 1999; accepted 14 September 1999

We found that p21 was also induced by p53 (Figure 1a, compare lanes 1 and 2). Furthermore, we found that mutant p53(R249S) was incapable of activating both TAP1 and p21 (Figure 1a, compare lanes 3 and 4), consistent with the fact that this tumor-derived p53 mutant is defective in transactivation. After normalization to the level of GAPDH mRNA, we estimated that the amount of TAP1 in cells expressing p53 was 4–6 times higher than in cells not expressing p53.

Since the p53 protein is stabilized and accumulates in cells following DNA damage (Ko and Prives, 1996), we determined whether TAP1 can be activated by DNA damage in the RKO colorectal carcinoma cell line, which contains an endogenous wild-type p53 gene (Nelson and Kastan, 1994). To this end, RKO cells were treated with camptothecin, doxorubicin, or actinomycin D. Camptothecin and doxorubicin are inhibitors of topoisomerase I and II, respectively, both of which induce double-strand DNA breaks (Nelson and Kastan, 1994). Actinomycin D inhibits transcription, but induces DNA damage at low concentrations (1–10 nM) (Nelson and Kastan, 1994). Northern blot analysis showed that TAP1 was induced in RKO cells treated with these DNA-damaging agents (Figure 1b). As expected, p21 was also activated (Figure 1b). After normalization to the level of GAPDH mRNA, we found that the amount of TAP1 expressed in RKO cells treated with these DNA-damaging agents was 4–8 times greater than in mock-treated cells.

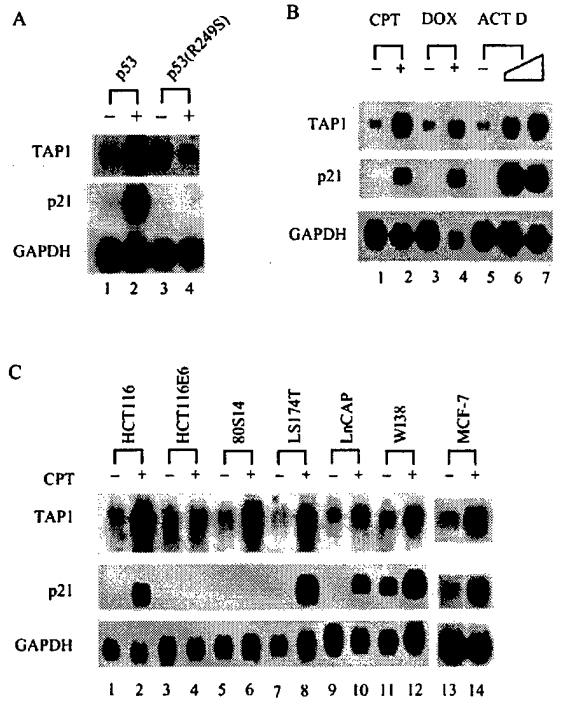


Figure 1 (a) Wild-type p53, but not p53 mutant, induces TAP1. A Northern blot was prepared using 10 μg of total RNA isolated from p53-3 or p53(R249S)-2 cells that were uninduced (−) or induced (+) to express wild-type p53 and mutant p53(R249S), respectively. (b) TAP1 is induced by three DNA-damaging agents in RKO cells. A Northern blot was prepared using 10 μg of total RNA isolated from untreated RKO cells (−) or cells treated (+) with 300 nM camptothecin (CPT), 1.0 μg/ml doxorubicin (DOX), 3.0 or 10 nM actinomycin D (ACT D). (c) TAP1 is induced by DNA damage in six cell lines that carry an endogenous wild-type p53 gene but not in one that is functionally p53-null. Northern blots were prepared using 10 μg of total RNA isolated from seven individual cell lines as indicated at the top of the figure, which were untreated (−) or treated (+) with 300 nM camptothecin for 24 h. The blots were probed with TAP1 cDNA, and then reprobed with p21 and GAPDH cDNAs, respectively

If TAP1 is a true cellular p53 target, TAP1 should be induced by p53 (i.e., DNA damage) in other cell lines that contain an endogenous wild-type p53 gene but not in cell lines that are p53-null. To this end, we tested seven different cell lines. HCT116, LS174T, LnCap, WI-38, and MCF7 each contain an endogenous wild-type p53 gene. 80S14 cell line is an HCT116 derivative that is p21-null (Waldman *et al.*, 1996), and HCT116E6 is an HCT116 derivative that contains human papillomavirus (HPV) oncoprotein E6. Since HPV E6 facilitates degradation of p53 (Ko and Prives, 1996), HCT116E6 is a p53-null-like cell line. These cells were treated with camptothecin and the levels of TAP1 and p21 determined by Northern blot analysis (Figure 1c). We found that both TAP1 and p21 were induced in cells containing wild-type p53 when treated with camptothecin (Figure 1c, lanes 1,2 and 7–14). Although p21 was not expressed in the p21-null 80S14 cells, TAP1 was still induced by DNA damage (Figure 1c, lanes 5 and 6), indicating that p53 can activate TAP1 independently of p21. In contrast, TAP1 was not induced in p53-null-like HCT116E6 cells (Figure 1c, lanes 3 and 4).

Since p73 is homologous to p53 (Kaghad *et al.*, 1997) and is capable of inducing p21 (Jost *et al.*, 1997; Kaghad *et al.*, 1997; Zhu *et al.*, 1998a), we wanted to determine whether TAP1 is a common cellular target of p53 and p73. To this end, we used three H1299 cell lines that inducibly express two alternatively spliced forms of wild-type p73, i.e., p73α and p73β, and one mutant p73α292, respectively (Zhu *et al.*, 1998a). We found that both TAP1 and p21 were induced by both wild-type p73α and p73β but not by mutant p73α292 (Figure 2a). Since both p53 and p73 are activators of transcription, they may cooperate to activate genes responsible for tumor suppression. To determine whether TAP1 is activated cooperatively by p53 and p73, TAP1 expression was examined in MCF7 cells that are either induced to express p73α, treated with camptothecin to induce p53, or both induced to express p73α and treated with camptothecin to induce p53 (Figure 2b). We found that TAP1 was up-regulated in MCF7 cells when treated with camptothecin (Figure 2b, compare lanes 1 and 2) or induced to express p73α (Figure 2b, compare lanes 1 and 4). After PhosphorImage quantitation, we found that TAP1 was induced 2.6-fold by either p53 or p73α. In contrast, TAP1 was induced 7.1-fold when both p53 and p73α were expressed in MCF7 cells (Figure 2b, lane 3). These results suggest that p73α and p53 (DNA damage) cooperate to activate TAP1 expression. We also found that p21 was activated cooperatively by p73α and DNA damage-induced p53 in MCF7 cells (Figure 2b).

Because TAP1 is one of the components required for the MHC class I antigen presentation pathway, we wanted to determine whether other genes in this pathway are regulated by p53. We examined five other genes by Northern blot analysis and found that TAP2, LMP2, LMP7 and MHC class I heavy chain

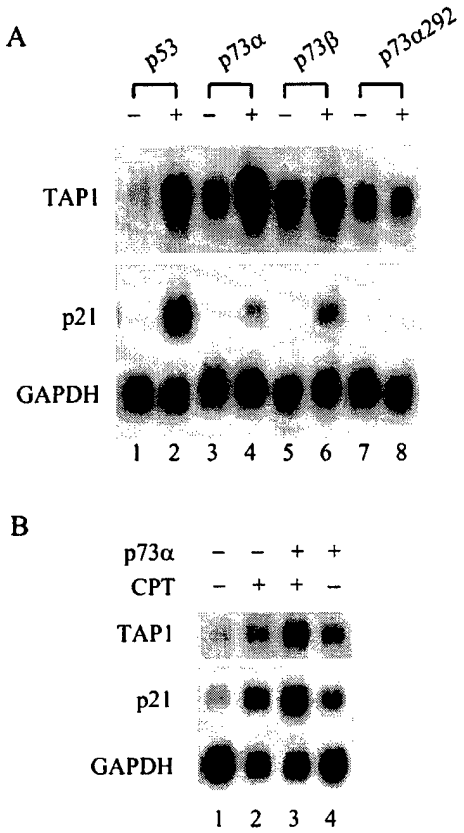


Figure 2 (**a**) Wild-type p73, but not mutant p73, is capable of inducing TAP1. A Northern blot was prepared using 10 μg of total RNA isolated from uninduced cells (lanes 1, 3, 5 and 7) or cells that were induced to express wild-type p53 (lane 2), p73α (lane 4), p73β (lane 6), or mutant p73α292 (lane 8). (**b**) p73 cooperates with DNA damage to activate TAP1 in MCF7 cells that carry an endogenous wild-type p53 gene. A Northern blot was prepared using 10 μg of total RNA isolated from MCF7 cells that were untreated (lane 1), treated with 300 nM camptothecin (CPT) to induce endogenous wild-type p53 (lane 2), induced to express exogenous p73α and treated with 300 nM camptothecin to induce endogenous wild-type p53 (lane 3), or induced to express exogenous p73α (lane 4). The blots were probed with TAP1, p21, and GAPDH cDNAs, respectively

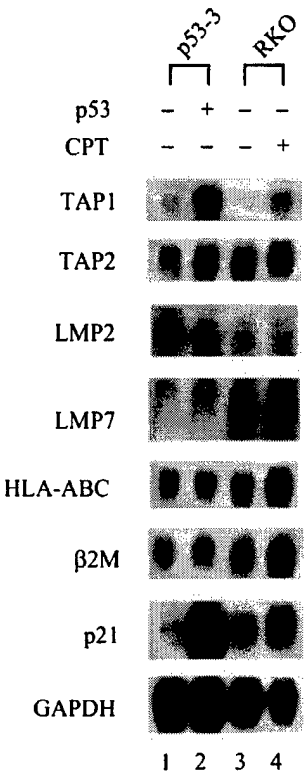


Figure 3 TAP1, but not other components in the MHC class I pathway, is induced by p53. Northern blots were prepared using 10 μg of total RNA isolated from p53-3 cells that were uninduced (–) or induced (+) to express exogenous wild-type p53, or from RKO cells that were untreated (–) or treated (+) with 300 nM camptothecin to induce endogenous wild-type p53 for 24 h. The blots were probed with TAP1, TAP2, LMP2, LMP7, HLA-ABC, β_2M, p21 and GAPDH cDNAs, respectively

HLA-ABC and light chain β_2M were expressed, but not significantly induced by p53 or DNA damage in p53-3 and RKO cells, respectively (Figure 3).

Next, we examined the level of TAP1 protein in p53-3 cells by Western blot analysis. We found that p53 expression resulted in the increase of TAP1 protein (Figure 4, compare lanes 1 and 2), consistent with p53 induction of TAP1 mRNA as analysed by Northern blot analysis (Figure 1a). p53-3 cells were also treated with 5, 15, 50, 100 and 500 U of IFNγ, a potent inducer of TAP1 (Stark *et al.*, 1998). We found that the TAP1 protein was efficiently induced with 15 U/ml of IFNγ (Figure 4, lane 3).

Identification of a specific p53-responsive element in the TAP1 gene

To define whether TAP1 is a true target of p53, we searched for a p53-responsive element in the genomic DNA sequence of the TAP1 gene. A potential p53-

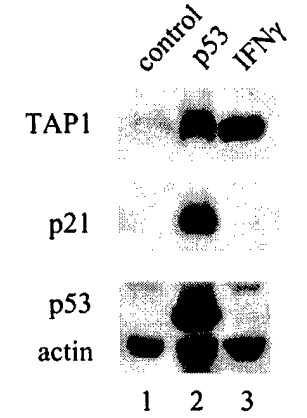


Figure 4 The TAP1 protein is increased in cells expressing p53 or treated with IFNγ. The levels of TAP1, p21, p53, and actin proteins in p53-3 cells that were untreated (lane 1), induced to express p53 (lane 2), or treated with 15 U/ml of IFNγ (lane 3), were assayed by Western blot analysis. The blots were probed with anti-TAP1 monoclonal antibody A148.3, anti-p21 monoclonal antibody, and a mixture of anti-p53 monoclonal antibody Pab1801 and anti-actin polyclonal antibody, respectively

binding site was found to be located approximately 300 nucleotides downstream of the TAP1 transcription start site (Beck *et al.*, 1992). This sequence (ggg cttg g*cc ctgccg gga cttg cct) has only one mismatch (G* instead of C/T) to the consensus p53-binding site (el-Deiry *et al.*, 1992). To analyse whether p53 binds to this sequence, a 59-bp DNA fragment containing this region was synthesized, ^{32}P-labeled, and used in an electrophoretic mobility shift assay (EMSA). We found that p53 interacts specifically with the potential p53-responsive element in the TAP1 gene (data not shown).

We further examined whether the potential p53-binding site is responsive to p53 *in vivo*. To do this, the potential p53-responsive element was cloned upstream of a minimal promoter and a luciferase reporter gene to generate the reporter vector TAP1-Fluc. The construct GADD45-Fluc, which contains a p53-responsive element from the GADD45 gene, a well-defined cellular p53 target, was used as a positive control as described previously (Chen *et al.*, 1995). We found that the luciferase activity for either TAP1-Fluc or GADD45-Fluc was markedly increased by wild-type p53 (Figure 5a), suggesting that p53 can bind to the p53-responsive elements from both the TAP1 and GADD45 genes. Interestingly, we observed that the increase in the luciferase activity by p53 for TAP1-Fluc was about five times greater than that for GADD45-Fluc (Figure 5a). This suggests that the p53-binding site in the TAP1 gene may have a higher affinity for p53 than the binding site in the GADD45 gene. Similarly, we found that the luciferase activity for TAP1-Fluc was increased by both p73α and p73β (Figure 5b). In contrast, the luciferase activity for TAP1-Fluc was not increased by the mutants p53(V143A), p53(R175H), p53(R249S), or p53(R273H) (Figure 5c), consistent with the observation that mutant p53(R249S) was incapable of inducing TAP1 (Figure 1a).

p53 induction of TAP1 leads to increased transport of MHC class I peptides

To determine whether induction of TAP1 by p53 can lead to increased transport of MHC class I peptides,

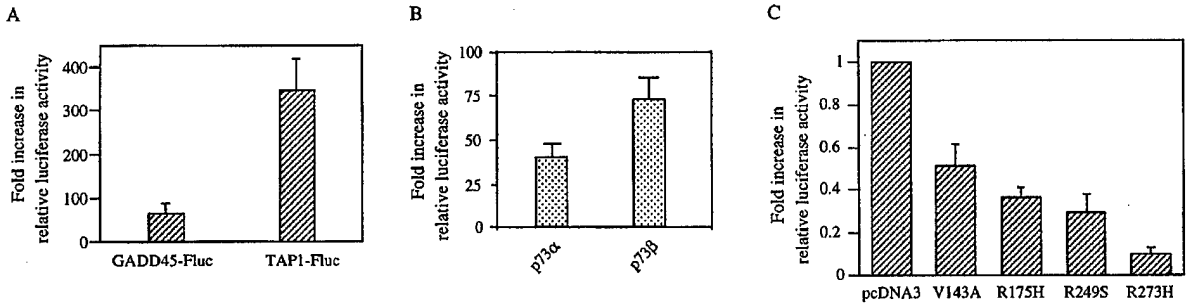


Figure 5 Wild-type p53 and p73 bind to the p53-responsive element *in vivo*. (**a**) The potential p53-binding site in the TAP1 gene is responsive to wild-type p53 *in vivo*. 5 μg of TAP1-Fluc or GADD45-Fluc was co-transfected into H1299 cells with 5 μg of pcDNA3 or a vector that expresses wild-type p53. The fold increase in relative luciferase activity is a product of the luciferase activity activated by p53 divided by that activated by pcDNA3. (**b**) The potential p53-binding site in the TAP1 gene is responsive to wild-type p73 *in vivo*. 5 μg of TAP1-Fluc was co-transfected into H1299 cells with 5 μg of pcDNA3 or a vector that expresses wild-type p73α or p73β. The fold increase in relative luciferase activity is calculated similarly to that in (**a**). (**c**) p53 mutants are unable to increase the luciferase activity for TAP1-Fluc. 5 μg of TAP1-Fluc was co-transfected into H1299 cells with 5 μg of pcDNA3 or a vector that expresses p53(V143A), p53(R175H), p53(R249S), or p53(R273H). The fold increase in relative luciferase activity was determined similarly to that in (**a**)

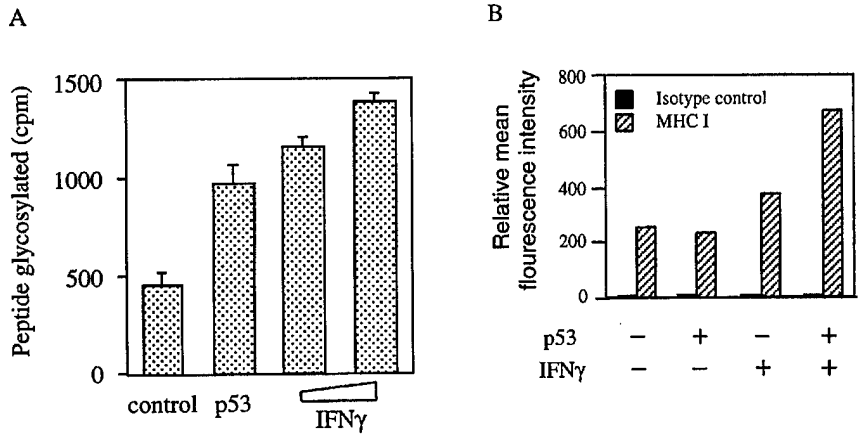


Figure 6 (**a**) p53 and IFNγ increases peptide transport capacity in p53-3 cells. p53-3 cells were uninduced, induced to express p53, or treated with 5 or 20 U/ml of IFNγ for 24 h. The extent of peptide glycosylation was then used to measure the relative peptide transport capacity in cells. (**b**) p53 cooperates with IFNγ to enhance the expression of surface MHC-peptide complexes. p53-3 cells that were uninduced or induced to express p53 were mock-treated or treated with 500 U/ml IFNγ for 48 h. The level of surface MHC-peptide complexes was determined by FACS analysis with anti-human HLA-ABC antibody B-H9. Mouse IgG1 was used as an isotype control

we performed peptide transport assays (Ma *et al.*, 1997). We found that the amount of glycosylated B27 peptide, a variant of an HLA-B27-binding, human histone 3 peptide, was significantly increased in p53-3 cells by p53 and IFNγ (Figure 6a). Similar results were obtained with A3 peptide, a variant of an HLA-A3-binding, HIV nef 7B peptide (data not shown). It should be noted that since IFNγ can also induce TAP2, the other key component for the transport of MHC class I peptides (Pamer and Cresswell, 1998), it is not surprising that IFNγ was more potent than p53 in enhancing the transport of B27 peptide (Figure 6a).

As MHC class I peptides are transported into the endoplasmic reticulum, they bind to assembled MHC class I molecules to form stable MHC-peptide complexes, which are subsequently expressed on the cell surface (Pamer and Cresswell, 1998). To determine whether p53 can increase the expression of surface MHC-peptide complexes on p53-3 cells, FACS analysis was performed. We found that the level of surface MHC-peptide complexes was not significantly increased by p53 (Figure 6b). This is not surprising since other abnormalities in the MHC class I pathway can inhibit MHC class I expression (Proffitt and Blair, 1997; Restifo *et al.*, 1993a). Indeed, the LMP7 gene, whose product is required for the generation of MHC class I peptides, was found to be expressed at an extremely low level in p53-3 cells (Figure 3). Consequently, the supply of cellular MHC class I peptides may be limited, which hinders the formation of stable MHC-peptide complexes. Therefore, we examined whether p53 can further increase MHC class I expression when p53-3 cells are treated with IFNγ to induce LMP7. We found that the level of MHC-peptides complexes expressed on IFNγ-treated cells was about 1.5 times higher than on untreated cells or cells expressing p53 (Figure 6b). However, when cells were both induced to express p53 and treated with IFNγ, the level of surface MHC-peptide complexes was 2.6 times greater than on untreated cells or cells expressing p53 alone (Figure 6b).

Since the LMP7 gene is highly expressed in the RKO cell line (Figure 3, LMP7 panel), we chose it to further determine whether p53 can enhance the transport of MHC class I peptides and expression of surface MHC-peptide complexes. As expected, when RKO cells were treated with camptothecin, the p53 protein was stabilized (Figure 7a, p53 panel), and subsequently, the TAP1 mRNA (Figure 7b, TAP1 panel) and protein (Figure 7a, TAP1 panel) upregulated. When RKO cells were treated with IFNγ, p53 was not stabilized (Figure 7a, p53 panel), but the TAP1 mRNA (Figure 7b, TAP1 panel) and protein (Figure 7a, TAP1 panel) were increased, suggesting that IFNγ can regulate the MHC class I pathway independently of p53 in RKO cells. To determine whether p53 is necessary for the enhanced expression of TAP1, we generated a derivative of the RKO cell line, RE6-26, which stably expresses HPV E6 oncoprotein. As a result, RE6-26 becomes a p53-null-like cell line. Indeed, p53 was undetectable in RE6-26 cells when treated with camptothecin (Figure 7a, compare lanes 4 and 5, p53 panel) and subsequently, the TAP1 mRNA (Figure 7b, TAP1 panel) and protein (Figure 7a, TAP1 panel) not induced. However, TAP1 was still induced in RE6-26 cells by IFNγ (Figure 7a, TAP1 panel; Figure 7b, TAP1 panel), suggesting that the IFNγ-regulated MHC class I pathway is not affected by the HPV E6 oncoprotein.

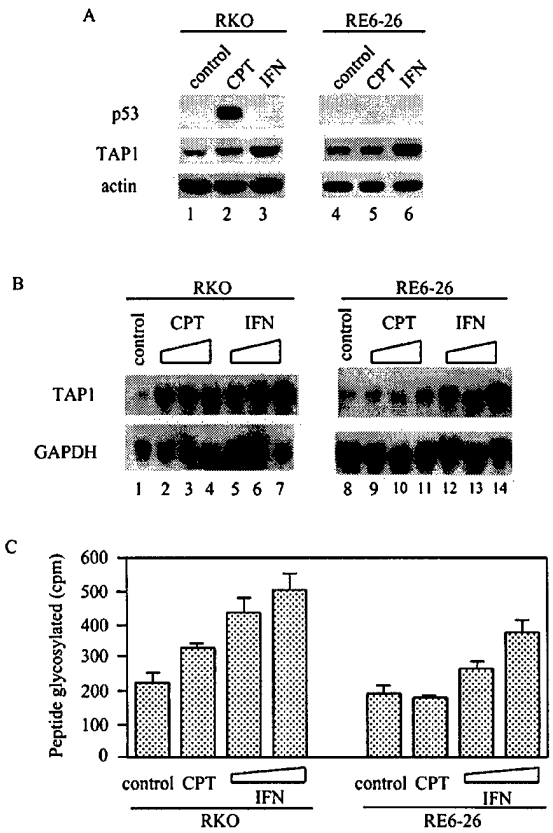


Figure 7 (a) p53 is required for the increased expression of TAP1 protein in RKO cells by DNA damage. The levels of TAP1, p53, and actin proteins in RKO and RE6-26 cells that were untreated (lanes 1 and 4), treated with 100 nM camptothecin (lanes 2 and 5), or 20 U/ml IFNγ (lanes 3 and 6), were assayed by Western blot analysis. The blots were probed with anti-p53 Pab1801, anti-TAP1 A148.3, and anti-actin polyclonal antibody, respectively. (b) p53 is required for the increased expression of TAP1 mRNA in RKO cells by DNA damage. Northern blots were prepared using 10 μg of total RNA isolated from RKO or RE6-26 cells that were untreated (lanes 1 and 8), treated with 50, 100, or 200 nM camptothecin (lanes 2–4 and 9–11), or treated with 10, 20, or 40 U/ml IFNγ (lanes 5–7 and 12–14) for 24 h. The blots were probed with TAP1 cDNA, and then reprobed with GAPDH cDNA. (c) p53 is required for the increased peptide transport capacity in RKO cells by DNA damage. Peptide transport assay was performed using RKO or RE6-26 cells that were untreated, treated with 100 nM camptothecin, or treated with 5 or 20 U/ml IFNγ for 24 h. The extent of peptide glycosylation was then used to measure the relative peptide transport capacity in cells

Next, we determined the peptide transport capacity in RKO and RE6-26 cells when treated with camptothecin or IFNγ. We found that the amount of glycosylated peptide was increased in RKO cells by both camptothecin and IFNγ (Figure 7c). In contrast, IFNγ, but not camptothecin, was capable of increasing the transport of MHC class I peptides in RE6-26 cells (Figure 7c).

To determine whether DNA damage can increase the expression of surface MHC-peptide complexes, RKO cells were treated with 0, 10, 25, 50, 250 and

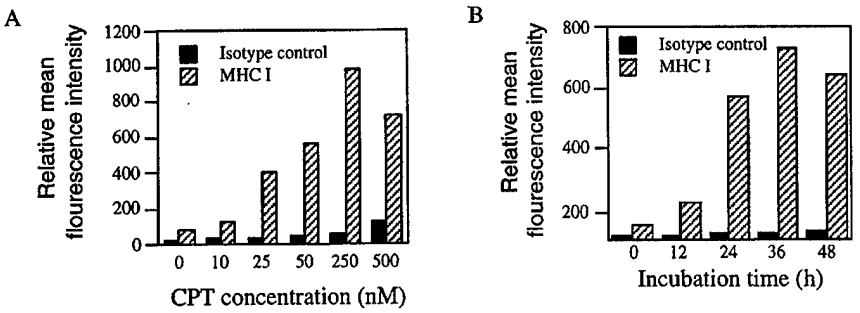


Figure 8 DNA damage increases the expression of surface MHC-peptide complexes on RKO cells when treated with camptothecin in dose- and time-dependent manners. (a) RKO cells were treated with 0, 10, 25, 50, 250 and 500 nM camptothecin for 24 h. (b) RKO cells were treated with 250 nM camptothecin for 0, 12, 24, 36 and 48 h. The level of surface MHC-peptide complexes was determined with anti-human HLA-ABC antibody B-H9. Mouse IgG1 was used as an isotype control

500 nM camptothecin for 24 h or treated with 250 nM camptothecin for 0, 12, 24, 36 and 48 h. We found that the level of surface MHC-peptide complexes was increased markedly in RKO cells by DNA damage in dose- and time-dependent manners (Figure 8a, b). In contrast, DNA damage had no effect on the MHC class I expression in RE6-26 cells (data not shown). These results suggest that p53 is responsible for the upregulation of the MHC class I pathway following DNA damage.

Discussion

In this study we have demonstrated that TAP1 can be induced by both p53 and several DNA-damaging agents. The induction of TAP1 by DNA damage is p53-dependent because TAP1 is not induced in cells when p53 is functionally null. We found that this induction is mediated by a p53-responsive element located 300 nucleotides downstream of the TAP1 transcription start site. Furthermore, the newly synthesized, p53-induced TAP1 protein is functional in increasing the transport of MHC class I peptides and subsequent expression of surface MHC-peptide complexes.

Since the MHC class I pathway is critical for host tumor surveillance (Pamer and Cresswell, 1998), tumor cells could evade tumor surveillance by acquiring mutations that inhibit the MHC class I pathway. Thus, mutation of one or more of the genes that encode key components for the MHC class I pathway would diminish or abrogate the host tumor surveillance. Indeed, the genes that encode the MHC heavy chain HLA-ABC and light chain β2M were found to be mutated in melanoma tumors (D'Urso *et al.*, 1991; Restifo *et al.*, 1993a). In adenovirus 12-transformed cells, the expression of the LMP2 gene was inhibited by adenoviral oncoproteins (Deiss and Kimchi, 1991; Proffitt and Blair, 1997). Interestingly, mutations that affect TAP1 occur frequently in a variety of human tumors (Amiot *et al.*, 1998; Chen *et al.*, 1996a; Cromme *et al.*, 1994; Kaklamanis *et al.*, 1995; Khanna *et al.*, 1998) and tumor cell lines (Alpan *et al.*, 1996; Johnsen *et al.*, 1998; Restifo *et al.*, 1993a; Vitale *et al.*, 1998; Wang *et al.*, 1998). Here we found that the tumor suppressor p53 is necessary for inducing TAP1 in cells following DNA damage. Thus, a dysfunctional p53 in more than 50% of human tumor cells would not induce TAP1 following genotoxic stress.

How does this novel activity of p53 relate to the central role of p53 in tumor suppression? p53 is a well-defined checkpoint protein in the cell cycle (Almog and Rotter, 1998; Ko and Prives, 1996; Levine, 1997). When cells are exposed to extracellular or intracellular stresses, for example, DNA damage, p53 is stabilized, resulting in cell cycle arrest, apoptosis, or differentiation. Cells suffering from DNA damage often express abnormal cellular proteins that need to be processed and presented on the cell surface (Old and Chen, 1998). These cells are then recognized by the host immune system, leading to their elimination. Our data suggest that p53 also activates the MHC class I pathway by inducing TAP1, which would facilitate this process. If tumor cells acquire additional mutations that inactivate p53, this process of tumor surveillance would be curtailed. Similarly, when oncogenic tumor viruses invade cells, viral proteins are expressed in cells, and then are processed and expressed on the cell surfaces by the MHC class I pathway, leading to elimination of the infected cells (McMichael, 1998; Ploegh, 1998). However, viral oncoproteins, such as HPV E6, adenoviral E1B, and hepatitis B virus (HBV) X, inactivate p53 (Ko and Prives, 1996), which in turn would abrogate the p53-dependent activation of TAP1. We have shown here that HPV E6 oncoprotein does just this in RKO and HCT116 cells. Subsequently, the infected cells would evade recognition by the host immune system and become transformed. Thus, we hypothesize that p53 may have a function in tumor surveillance and inactivation of p53 may be one mechanism that tumor cells use to evade host tumor surveillance.

The MHC class I pathway has been found to be defective in several neuroblastoma cell lines (Cheng *et al.*, 1996), which also carry a hemizygous deletion of a 9 cM interval on chromosome 1p35-36.1 where the p73 gene is located (Kaghad *et al.*, 1997). Since p73 is expressed from only one allele in some cells due to genomic imprinting (Kaghad *et al.*, 1997), a hemizygous deletion of the expressible allele would result in total loss of p73 expression. In this study, we found that p73 is capable of activating the TAP1 gene. Thus,

consistent with the previous observation, loss of p73 may be responsible for down-regulation of the MHC class I pathway in some neuroblastoma cells.

IFN-γ is the most potent inducer of the MHC class I pathway (Stark *et al.*, 1998). Upon binding to its receptor, IFNγ activates the Jak/Stat signaling pathway, leading to induction of at least two groups of transcriptional activators, i.e., the IFN regulatory factors (IRFs) and the class II transactivator (CIITA). IRFs bind to the IFN-stimulated response element (ISRE) and activate several genes in the MHC class I pathway, including the TAP1 gene (Pamer and Cresswell, 1998; Stark *et al.*, 1998). CIITA binds to the site α in the MHC class I heavy chain genes and activates HLA-ABC expression (Gobin *et al.*, 1997; Martin *et al.*, 1997). Since the induction of TAP1 by IFNγ occurs in H1299 cells that are p53-null (Figure 4), the regulation of the MHC class I pathway by IFNγ is independent of p53. A recent report showed that IFNγ-insensitive $p53^{-/-}$ mice develop tumors more rapidly with a broader spectrum of tumors when compared to either $p53^{-/-}$ mice or IFNγ-insensitive mice individually (Kaplan *et al.*, 1998). Furthermore, we found that p53 can cooperate with IFNγ to activate the MHC class I pathway. Thus, it is likely that tumor cells lacking both p53 and an IFNγ response would be defective in the MHC class I antigen presentation pathway, and such cells would become less immunogenic.

Materials and methods

Cell culture

H1299, HCT116, LS174T, LnCap, MCF-7 and WI-38 cell lines were purchased from American Type Culture Collection. RKO cells were cultured as described (Nelson and Kastan, 1994). 8OS14 cell line was cultured as described (Waldman *et al.*, 1996). RE6-26 and HCT116E6 are derivatives of RKO and HCT116, respectively, which were stably transfected with the E6 gene from human papilloma virus (HPV) 16 (Munger *et al.*, 1989). p53-3 and p53(R249S)-2 cell lines, derivatives of H1299 that inducibly express wild-type p53 and p53(R249S), respectively, were cultured as described (Chen *et al.*, 1996b). The H1299 cell lines that inducibly express p73α, p73α292 and p73β are p73α-22, p73α292-20 and p73β-9, respectively, as previously described (Zhu *et al.*, 1998a). The MCF7 cell line, which expresses tet-VP16 for generation of tetracycline inducible cell lines, was purchased from ClonTech (Palo Alto, CA, USA). MCF7 cell lines that express inducible proteins of interest were generated as previously described (Chen *et al.*, 1996b). Camptothecin, doxorubicin, and actinomycin D were purchased from Sigma (St. Louis, MC, USA). Human recombinant IFNγ was purchased from Boehringer Mannheim Biochemical (Germany).

RNA isolation, cDNA subtraction assay, and Northern blot analysis

Poly(A)$^+$ RNA was isolated from p53-3 cells using mRNA purification kit (Pharmacia, Piscataway, NJ, USA). Total RNA was isolated from cells using Trizol reagents (Life Technologies, Inc., Gaithersburg, MD, USA). cDNA subtraction assay was performed using ClonTech PCR-Select cDNA Subtraction kit (ClonTech, Palo Alto, CA, USA). Northern blot analysis was performed as described previously (Zhu *et al.*, 1998a). p21 and GAPDH probes were prepared as described previously (Zhu *et al.*, 1998b). TAP1 probe, a 800-bp *Sma*I–*Hind*III fragment, was prepared from human TAP1 cDNA. LMP2 and TAP2 probes were generated by RT–PCR as described previously (Restifo *et al.*, 1993a). HLA-ABC probe was prepared from mouse H2-K^b cDNA. β_2M cDNA probe (GenBank # AA143790) and LMP7 cDNA probe (AA147042) were purchased from Genome System Inc. (St. Louis, MO, USA).

Electrophoretic mobility shift assay (EMSA) and luciferase assay

Purification of the p53 protein and EMSA were performed as described previously (Chen *et al.*, 1993). The EMSA probe was a 59-bp fragment containing a potential p53-binding site (underlined) in the TAP1 gene: 5′-atcgacgtaagcttctg-cagggcttgg*ccctgccgsggacttgcctagatctacgt-3′. For luciferase assay, the fragment was cloned upstream of a minimal c-*fos* promoter and a firefly luciferase reporter gene (Johansen and Prywes, 1994), and the resulting construct designated TAP1-Fluc. GADD45-Fluc was described previously (Chen *et al.*, 1995). TAP1-Fluc or GADD45-Fluc was co-transfected into H1299 cells with control vector pcDNA3 or a vector that expresses wild-type p53, p53(V143A), p53(R175H), p53(R249S), p53(R273H), p73α or p73β. Dual luciferase assay was performed according to the manufacturer's instructions (Promega).

Western blot analysis

Western blot analysis was performed as described previously (Zhu *et al.*, 1998b). Anti-human TAP1 monoclonal antibody, Ab148.3, was kindly provided by Dr B Seliger (Meyer *et al.*, 1994). Antibodies against p53, p21, actin were described previously (Zhu *et al.*, 1998a).

Peptide, peptide labeling and peptide transport assay

Two MHC class I peptides were synthesized by Molecular Biology Core Facility (Medical College of Georgia) for use in the transport assay. These were: B27, a variant of an HLA-B27-binding, human histone 3 peptide (RRYQ*N*STEL), where Asn is substituted for Lys (Ma *et al.*, 1997); and A3, a variant of an HLA-A3-binding, HIV nef 7B peptide (QVPLR*N*MTYK), where Asn is substituted for Pro (Ma *et al.*, 1997). The peptides were labeled with Na ^{125}I (Amersham Pharmacia) and purified through a sephadex G-25 column. The specific activity of the labeled peptides was approximately 100 c.p.m./fmol. Transport assay was performed as previously described (Ma *et al.*, 1997).

FACS analysis

FACS analysis was performed as previously described (Ma *et al.*, 1997). FITC-labeled mouse anti-human HLA-ABC monoclonal antibody B-H9 was purchased from BioSource International (Carmarillo, CA, USA). FITC-labeled mouse IgG1 monoclonal antibody was purchased from PharMingen (San Diego, CA, USA). The relative amount of the surface MHC-peptide complexes is measured by the relative mean fluorescence intensity from FITC-labeled mouse anti-human HLA-ABC monoclonal antibody.

Acknowledgments
We are grateful to Drs R Markowitz, W Dynan, D Munn, M Iwashima and A Mellor for their critical reading of this manuscript and/or suggestions. We would like to thank H Ploegh (MIT, MA) for human TAP1 cDNA, J Trowsdale (University of Cambridge, UK) for TAP1 genomic DNA, B Seliger (Johannes Gutenberg-Universität, Germany) for anti-TAP1 antibody, D Dransfield (Medical College of

Georgia, GA, USA) for HCT116 and LST174T cell lines, B Vogelstein (Johns Hopkins University, MD, USA) for 8OS14 cell line, and A Mellor (Medical College of Georgia, GA, USA) for H2-K^b cDNA. This work is supported in part by grants from the National Institute of Health (CA76069 and CA81237) and DOD Army Breast Cancer Program (DAMD17-97-1-7019).

References

Almog N and Rotter V. (1998). *Biochim. Biophys. Acta.*, **1378,** R43–R54.

Alpan RS, Zhang M and Pardee AB. (1996). *Cancer Res.*, **56,** 4358–4361.

Amiot L, Onno M, Lamy T, Dauriac C, Le Prise PY, Fauchet R and Drenou B. (1998). *Br. J. Haematol.*, **100,** 655–663.

Beck S, Kelly A, Radley E, Khurshid F, Alderton RP and Trowsdale J. (1992). *J. Mol. Biol.*, **228,** 433–441.

Chen HL, Gabrilovich D, Tampe R, Girgis KR, Nadaf S and Carbone DP. (1996a). *Nat. Genet.*, **13,** 210–213.

Chen X. (1991). *Mol. Med. Today* **5,** 387–392.

Chen X, Bargonetti J and Prives C. (1995). *Cancer Res.*, **55,** 4257–4263.

Chen X, Farmer G, Zhu H, Prywes R and Prives C. (1993). *Genes Dev.*, **7,** 1837–1849.

Chen X, Ko LJ, Jayaraman L and Prives C. (1996b). *Genes Dev.*, **10,** 2438–2451.

Cheng NC, Chan AJ, Beitsma MM, Speleman F, Westerveld A and Versteeg R. (1996). *Hum. Mol. Genet.*, **5,** 309–317.

Cromme FV, Airey J, Heemels MT, Ploegh HL, Keating PJ, Stern PL, Meijer CJ and Walboomers JM. (1994). *J. Exp. Med.*, **179,** 335–340.

D'Urso CM, Wang ZG, Cao Y, Tatake R, Zeff RA and Ferrone S. (1991). *J. Clin. Invest.*, **87,** 284–292.

Deiss LP and Kimchi A. (1991). *Science,* **252,** 117–120.

el-Deiry WS, Kern SE, Pietenpol JA, Kinzler KW and Vogelstein B. (1992). *Nat. Genet.*, **1,** 45–49.

el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW and Vogelstein B. (1993). *Cell,* **75,** 817–825.

Gobin SJ, Peijnenburg A, Keijsers V and van den Elsen PJ. (1997). *Immunity.*, **6,** 601–611.

Hermeking H, Lengauer C, Polyak K, He TC, Zhang L, Thiagalingam S, Kinzler KW and Vogelstein B. (1997). *Mol. Cell.*, **1,** 3–11.

Hollstein M, Sidransky D, Vogelstein B and Harris CC. (1991). *Science,* **253,** 49–53.

Johansen FE and Prywes R. (1994). *Mol. Cell. Biol.*, **14,** 5920–5928.

Johnsen A, France J, Sy MS and Harding CV. (1998). *Cancer Res.*, **58,** 3660–3667.

Jost CA, Marin MC and Kaelin Jr WG. (1997). *Nature,* **389,** 191–194.

Kaghad M, Bonnet H, Yang A, Creancier L, Biscan JC, Valent A, Minty A, Chalon P, Lelias JM, Dumont X, Ferrara P, McKeon F and Caput D. (1997). *Cell,* **90,** 809–819.

Kaklamanis L, Leek R, Koukourakis M, Gatter KC and Harris AL. (1995). *Cancer Res.*, **55,** 5191–5194.

Kaplan DH, Shankaran V, Dighe AS, Stockert E, Aguet M, Old LJ and Schreiber RD. (1998). *Proc. Natl. Acad. Sci. USA,* **95,** 7556–7561.

Khanna R, Busson P, Burrows SR, Raffoux C, Moss DJ, Nicholls JM and Cooper L. (1998). *Cancer Res.*, **58,** 310–314.

Ko LJ and Prives C. (1996). *Genes Dev.*, **10,** 1054–1072.

Levine AJ. (1997). *Cell,* **88,** 323–331.

Ma W, Lehner PJ, Cresswell P, Pober JS and Johnson DR. (1997). *J. Biol. Chem.*, **272,** 16585–16590.

Martin BK, Chin KC, Olsen JC, Skinner CA, Dey A, Ozato K and Ting JP. (1997). *Immunity,* **6,** 591–600.

McMichael A. (1998). *Cell,* **93,** 673–676.

Meyer TH, van Endert PM, Uebel S, Ehring B and Tampe R. (1994). *FEBS Lett.*, **351,** 443–447.

Miyashita T, Krajewski S, Krajewska M, Wang HG, Lin HK, Liebermann DA, Hoffman B and Reed JC. (1994). *Oncogene,* **9,** 1799–1805.

Munger K, Phelps WC, Bubb V, Howley PM and Schlegel R. (1989). *J. Virol.*, **63,** 4417–4421.

Nelson WG and Kastan MB. (1994). *Mol. Cell. Biol.*, **14,** 1815–1823.

Old LJ and Chen YT. (1998). *J. Exp. Med.*, **187,** 1163–1167.

Pamer E and Cresswell P. (1998). *Annu. Rev. Immunol.*, **16,** 323–358.

Ploegh HL. (1998). *Science,* **280,** 248–253.

Polyak K, Xia Y, Zweier JL, Kinzler KW and Vogelstein B. (1997). *Nature,* **389,** 300–305.

Proffitt JA and Blair GE. (1997). *FEBS Lett.*, **400,** 141–144.

Restifo NP, Esquivel F, Kawakami Y, Yewdell JW, Mule JJ, Rosenberg SA and Bennink JR. (1993a). *J. Exp. Med.*, **177,** 265–272.

Restifo NP, Kawakami Y, Marincola F, Shamamian P, Taggarse A, Esquivel F and Rosenberg SA. (1993b). *J. Immunother.*, **14,** 182–190.

Stark GR, Kerr IM, Williams BR, Silverman RH and Schreiber RD. (1998). *Annu. Rev. Biochem.*, **67,** 227–264.

Vitale M, Rezzani R, Rodella L, Zauli G, Grigolato P, Cadei M, Hicklin DJ and Ferrone S. (1998). *Cancer Res.*, **58,** 737–742.

Waldman T, Lengauer C, Kinzler KW and Vogelstein B. (1996). *Nature,* **381,** 713–716.

Wang Z, Seliger B, Mike N, Momburg F, Knuth A and Ferrone S. (1998). *Cancer Res.*, **58,** 2149–2157.

Zhu J, Jiang J, Zhou W and Chen X. (1998a). *Cancer Res.*, **58,** 5061–5065.

Zhu J, Zhou W, Jiang J and Chen X. (1998b). *J. Biol. Chem.*, **273,** 13030–13036.

Oncogene (2000) 19, 1843–1848
© 2000 Macmillan Publishers Ltd All rights reserved 0950–9232/00 $15.00
www.nature.com/onc

SHORT REPORT

Dickkopf-1, an inhibitor of the Wnt signaling pathway, is induced by p53

Jian Wang[1], Jiang Shou[1] and Xinbin Chen*,[1]

[1]*Institute of Molecular Medicine and Genetics, Medical College of Georgia, Augusta, Georgia, GA 30912, USA*

Dickkopf-1 (Dkk-1), a secreted glycoprotein, has been found to be necessary and sufficient for inducing amphibian head formation. Interestingly, the mechanism by which Dkk-1 does this is the ability of Dkk-1 to antagonize the Wnt signaling pathway. Wnt, itself a proto-oncoprotein, can promote cell proliferation and transformation when mutated or overexpressed, leading to tumor formation. p53 is a tumor suppressor and loss of p53 function accelerates mammary tumorigenesis by Wnt. In this study, we found that Dkk-1 is induced by wild-type p53 but not mutant p53(R249S). In addition, DNA damage upregulates Dkk-1 in cell lines that harbor an endogenous wild-type p53 gene but not in cell lines that are p53-null or harbor an endogenous mutant p53 gene. We also found a potential p53 responsive element located approximately 2100 nucleotides upstream of the Dkk-1 transcription start site and we show that p53 binds specifically to this element both *in vitro* and *in vivo*. Furthermore, we have established several cell lines derived from H1299 lung carcinoma and U118 glioma cells that inducibly express Dkk-1 under a tetracycline-regulated promoter. We found that Dkk-1 has no effect on proliferation of cells that are not transformed by Wnt. Taken together, these results suggest that Dkk-1 may mediate p53 tumor suppression by antagonizing the Wnt signaling pathway. *Oncogene* (2000) **19,** 1843–1848.

Keywords: p53; Dickkopf-1; Wnt

The Wnt genes, encoding a large family of secreted, cysteine-rich glycosylated proteins, are evolutionarily conserved among diverse organisms such as *Homo sapiens*, *Mus musculus*, *Xenopus laevis*, *Drosophila melanogaster*, and *Caenorhabditis elegans* (Brown and Moon, 1998; Nusse and Varmus, 1992; Wodarz and Nusse, 1998). Genetic studies have demonstrated that the Wnt proteins serve as intercellular signaling molecules and play key roles in embryogenesis, segment polarity, central nervous system (CNS) patterning, and the control of asymmetric cell divisions (Wodarz and Nusse, 1998). Wnt signaling events are initiated by the binding of Wnt to its receptor, Frizzled (Krasnow *et al.*, 1995; Wong and Adler, 1993), which leads to activation of the Dishevelled protein (Klingensmith *et al.*, 1994; Krasnow *et al.*, 1995; Theisen *et al.*, 1994). The activated Dishevelled protein enhances the phosphorylation of glycogen synthase kinase (GSK) (Cook *et al.*, 1996), which inhibits the ability of GSK to phosphorylate β-catenin, leading to increased stability and accumulation of β-catenin (Munemitsu *et al.*, 1996; Pai *et al.*, 1997; Yost *et al.*, 1996). β-catenin can interact with members of T cell factor (TCF)/lymphoid enhancer factor (LCF) family in the nucleus, which regulates Wnt target genes necessary for development (Wodarz and Nusse, 1998).

Abnormal activation of the Wnt signaling pathway can lead to developmental catastrophe, such as duplication of the embryonic axis and subsequent induction of two-headed embryos in *Xenopus laevis*, and tumor formation in the mouse and human (Brown and Moon, 1998; Nusse and Varmus, 1992; Wodarz and Nusse, 1998). Recent studies in *Xenopus* embryos have identified at least four families of inhibitors of the Wnt signaling pathway, that is, Frizzled-related protein (FRP), Cerberus, Wnt-inhibitory factor-1 (WIF-1), and Dickkopf-1 (Dkk-1). Cerberus and WIF-1 physically interact with and inhibit Wnt (Glinka *et al.*, 1997; Hsieh *et al.*, 1999; Piccolo *et al.*, 1999). FRP inhibits the Wnt signaling pathway by physically associating with both Wnt and its receptor, Frizzled (Bafico *et al.*, 1999; Finch *et al.*, 1997; Leyns *et al.*, 1997; Wang *et al.*, 1997). Dkk-1 inhibits Wnt-mediated axis duplication in *Xenopus* (Glinka *et al.*, 1998). In addition, by inhibiting the Wnt signaling pathway, Dkk-1 is sufficient and necessary for head induction (Glinka *et al.*, 1998). Furthermore, Dkk-1 suppresses the ability of Wnt to promote cell proliferation (Fedi *et al.*, 1999). However, the mechanism by which Dkk-1 inhibits the Wnt signaling pathway and how Dkk-1 is regulated are still not clear.

p53 is a checkpoint protein. A large body of evidence suggests that p53 plays an important role in the regulation of numerous processes including cell cycle progression, differentiation, and apoptosis (Argarwal *et al.*, 1998; Almog and Rotter, 1997; Ko and Prives, 1996; Levine, 1997). Loss or mutation of p53 in some tumors has been correlated with a marked decrease of apoptosis and/or with a marked increase of cell proliferation. As a result, p53 deficiency can convert a slow growing tumor to a rapidly growing one (Howes *et al.*, 1994; Pan and Griep, 1995; Symonds *et al.*, 1994). Indeed, tumors appear at an earlier age in Wnt-1 and *ras* transgenic mice lacking p53 than in animals carrying one or both alleles of the p53 gene (Donehower *et al.*, 1995; Hundley *et al.*, 1997). Interestingly, the early onset of tumors in the Wnt-1 and *ras* transgenic mice is due to enhanced tumor cell proliferation but not decreased apoptosis in the absence of p53. Thus, p53 activities are necessary for inhibiting the acquired growth potential of tumor cells conferred by Wnt and *ras* (Hundley *et al.*, 1997; Jones *et al.*, 1997).

p53 transcriptional activity is necessary for tumor suppression (Chen, 1999; el-Deiry, 1998; Ko and Prives, 1996; Levine, 1997). p53 directly binds to DNA in a sequence specific manner and transactivates

*Correspondence: X Chen
Received 23 November 1999; revised 21 January 2000; accepted 31 January 2000

cellular target genes. A number of cellular genes has been found to be induced by p53. Among these are p21 (el-Deiry *et al*., 1993), GADD45 (Kastan *et al*., 1992), BAX (Miyashita *et al*., 1994), MDM2 (Wu *et al*., 1993), BTG2 (Rouault *et al*., 1996), PIGs (Polyak *et al*., 1997), 14-3-3σ (Hermeking *et al*., 1997), IGFBP3 (Buckbinder *et al*., 1995), PI(3)K regulatory subunit p85 (Yin *et al*., 1998), KILLER/DR5 (Wu *et al*., 1997), and TAP I (Zhu *et al*., 1999). p21, 14-3-3σ, GADD45, and BTG2 have been shown to be capable of mediating p53-dependent cell cycle arrest (Chen *et al*., 1996; el-Deiry *et al*., 1993; Hermeking *et al*., 1997; Rouault *et al*., 1996; Wang *et al*., 1999) while BAX, p85, IGFBP3, KILLER/DR5 and PIGs may mediate apoptosis (Buckbinder *et al*., 1995; Miyashita *et al*., 1994; Polyak *et al*., 1997; Wu *et al*., 1997; Yin *et al*., 1998). Recently, we found that TAP1 is specifically induced by both p53 and p73, which leads to enhanced transport of MHC class I peptides, suggesting that tumor surveillance can be mediated by the p53 family tumor suppressor proteins (Zhu *et al*., 1999).

In our ongoing effort to identify novel p53 target genes, the ClonTech PCR-Select cDNA Subtraction Assay was performed using mRNA isolated from p53-3, a derivative of the H1299 cell line that inducibly expresses p53 under a Tet-Off tetracycline-regulated promoter (Chen *et al*., 1996). Several cDNA fragments that may represent gene activated by p53 were isolated. After DNA sequencing and comparison with known sequences in GenBank, one subtracted cDNA fragment was found to be derived from the Dkk-1 gene. To confirm that Dkk-1 is specifically induced by wild-type but not mutant p53, Northern blot analysis was performed using Dkk-1 cDNA as probe. We found that Dkk-1 was induced by p53 in p53-3 cells (Figure 1a, compare lanes 1 and 2). As a control, we tested expression of p21, a well-defined cellular p53 target gene (el-Deiry *et al*., 1993). We found that p21 was also induced by p53 (Figure 1a, compare lanes 1 and 2). Furthermore, we found that mutant p53(R249S) was incapable of inducing either Dkk-1 or p21 (Figure 1a, compare lanes 3 and 4), consistent with the fact that

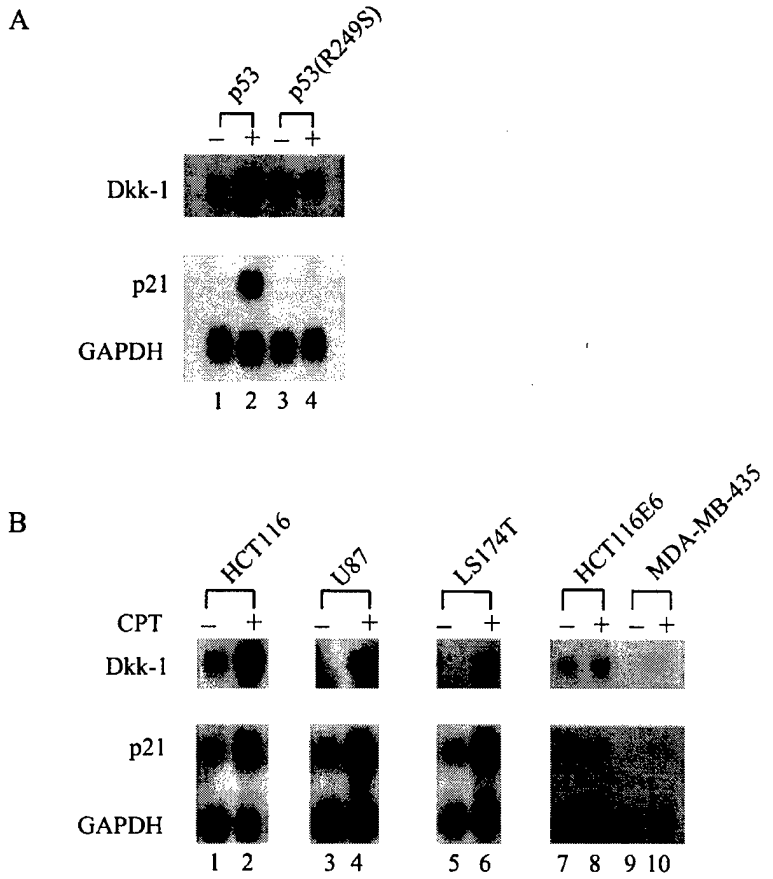


Figure 1 Upregulation of Dkk-1 by p53. (**a**) Wild-type p53, but not p53 mutant, induces Dkk-1. A Northern blot was prepared using 10 μg of total RNA isolated from p53-3 or p53(R249S)-4 cells that were uninduced (−) or induced (+) to express wild-type p53 and mutant p53(R249S), respectively. The blot was probed with Dkk-1 cDNA, and then reprobed with both p21 and GAPDH cDNAs. (**b**) Dkk-1 is induced by DNA damage in cell lines that carry an endogenous wild-type p53 gene but not in cells that are p53-null-like or contain an endogenous mutant p53 gene. Northern blots were prepared using 10 μg of total RNA isolated from HCT116, U87, LS174T, HCT116E6 or MDA-MB-435 cells that were untreated (−) or treated (+) with 300 nM camptothecin for 24 h. The blots were probed with Dkk-1 cDNA, and then reprobed with p21 and GAPDH cDNAs. Northern blot analysis was performed as described previously (Zhu *et al*., 1998). p21 and GAPDH probes were prepared as described previously (Zhu *et al*., 1998). Dkk-1 probe, a 600 bp *Hind*III fragment, was prepared from human Dkk-1 cDNA

this tumor-derived p53 mutant is defective in transactivation (Friedlander *et al.*, 1996). After normalization to the level of GAPDH mRNA, we estimated that the amount of Dkk-1 in cells expressing p53 was up to 6–8 times higher than in cells not expressing p53.

DNA damage stabilizes and activates p53, leading to induction of p53 target genes (Giaccia and Kastan, 1998; Ko and Prives, 1996; Levine, 1997). If Dkk-1 is a true p53 target, it would be induced by DNA damage in cells that contain an endogenous wild-type p53 gene but not in cell lines that are p53-null or contain an endogenous mutant p53 gene. To this end, we tested five cell lines using the DNA damaging agent camptothecin, which is an inhibitor of topoisomerase I and can induce double strand DNA breaks (Nelson and Kastan, 1994). These cells were treated with camptothecin and the levels of Dkk-1 and p21 determined by Northern blot analysis (Figure 1b). We found that both Dkk-1 and p21 were induced in camptothecin-treated HCT116, LS174T, and U87 cells,

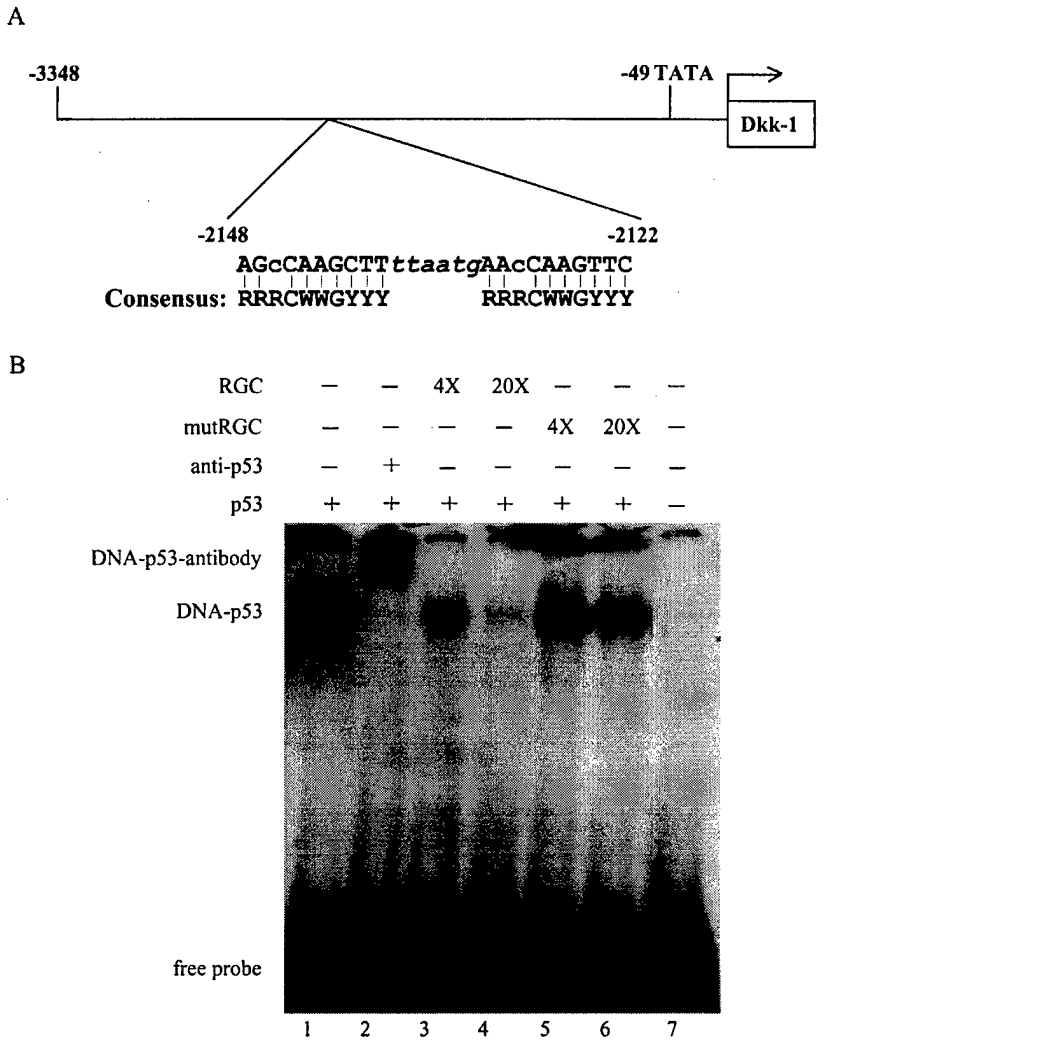


Figure 2 Identification of a p53-responsive element in the Dkk-1 gene. (**a**) Schematic representation of the Dkk-1 genomic DNA structure. The position of the potential Dkk-1 transcription start site and a potential p53 responsive element are indicated. Shown below the genomic structure are the sequence of the potential p53 responsive element and the previously characterized p53 consensus responsive element (el-Deiry *et al.*, 1992; Funk *et al.*, 1992). R represents purine, Y pyrimidine, and W adenine or thymidine. (**b**) p53 binds specifically to the potential p53 responsive element *in vitro*. A 32-bp oligonucleotide fragment containing the potential p53 responsive element in the Dkk-1 gene with the following sequence; 5′-AGCTTAGCCAAGCTTTTAATGAACCAAGTTCA-3′ (top strand) and 5′-GATCTGAACTTGGTTCATTAAAAGCTTGGCTA-3′ (bottom strand), was labeled with α-^{32}P-dCTP. 5 ng of the labeled probe DNA was added to a mixture [20 mM HEPES (pH 7.9), 25 mM KCl, 0.1 mM EDTA, 10% glycerol, 2 mM $MgCl_2$, 2 mM spermidine, 0.5 mM DTT, 0.025% NP-40, 100 ng double-stranded poly(dI:dC), and 2 μg BSA] containing 20 ng of p53 protein. The p53 protein was expressed in a baculovirus expression system and affinity-purified using anti-p53 monoclonal antibody Pab421. The p53-DNA complex was resolved in a 4% polyacrylamide gel. For 'supershifting' the p53-DNA complex, 1 μg of anti-p53 monoclonal antibody Pab1801 was added in the reaction in lane 2. For competition assays, unlabeled wild-type RGC (20 and 100 ng) or mutant RGC (20 and 100 ng) were added to the reaction run in lanes 3–4 and 5–6, respectively

which all contain wild-type p53 (Figure 1b, lanes 1–6). In contrast, Dkk-1 was not induced in p53-null-like HCT116E6 cells and MDA-MB-435 cells that carry an endogenous mutant p53 gene (Figure 1b, lanes 7–10). It should be noted that in HCT116 cells, the basal level of Dkk-1 expression is much higher than that in U87 and LS174T cells. Since several potential binding sites for other transcription factors in addition to p53 are present in the promoter region of the Dkk-1 gene (more discussion below), it is possible that one or more transcription factors may be responsible for the high basal level of Dkk-1 expression in HCT116 cells.

To determine whether Dkk-1 is regulated transcriptionally by p53, we searched for a p53-responsive element in the Dkk-1 genomic DNA sequence. Using Dkk-1 cDNA as probe, we screened a human bacterial artificial chromosome (BAC) library and obtained a genomic DNA clone that contains the human Dkk-1 gene. An approximately 3.4-kb DNA in the promoter region of the Dkk-1 gene was sequenced. We found a potential p53-binding site located approximately 2.1-kb upstream of the Dkk-1 transcription start site (Figure 2a). This sequence (AGc CAAG CTT TTAATG AAc CAAG TTC) has two mismatches (cytosine in lower case instead of guanine or adenine) in the non-critical positions within the consensus p53-binding site (el-Deiry *et al.*, 1992; Funk *et al.*, 1992).

To analyse whether p53 binds to the potential p53 responsive element, a 32-bp DNA fragment containing this element was synthesized, ^{32}P-labeled, and used in an electrophoretic mobility shift assay (EMSA). We found that when the purified p53 protein was mixed with the DNA fragment, a complex that presumably contained both p53 and DNA was detected (Figure 2b, lane 1). The complex was 'supershifted' with the anti-p53 monoclonal antibody Pab1801 (lane 2). We also used two other DNA fragments that contain either a wild-type or mutant p53-binding site from the ribosomal gene cluster (RGC) (Kern *et al.*, 1991) as competitors. The unlabeled wild-type RGC competed with the ^{32}P-labelled 32-bp DNA fragment from the Dkk-1 gene and inhibited the formation of the p53-DNA complex in a dose-dependent manner (lanes 3 and 4). In contrast, mutant RGC was unable to compete (lanes 5 and 6). These results indicate that p53 interacts specifically with the potential p53 responsive element in the Dkk-1 gene.

We further examined whether the potential p53-binding site is responsive to p53 *in vivo*. To do this, the potential p53 responsive element was cloned upstream of a minimal c-*fos* promoter (Johansen and Prywes, 1994) and a luciferase reporter gene to generate a reporter vector, designated Dkk-1-Fluc. We also substituted four nucleotides in the potential p53 responsive element predicted to be critical for p53-binding (shown in lower case) (AGC aAAt CTT T-TAATG AAC aAAt TTC). We then generated a reporter vector designated mut-Dkk-1-Fluc. Dkk-1-Fluc or mut-Dkk-1-Fluc was cotransfected into H1299 cells with either pcDNA3 control vector or a vector that expresses one of the following: wild-type p53, p53(V143A), p53(R175H), p53(R249S), and p53 (R273H). We found that the luciferase activity for Dkk-1-Fluc was markedly increased by wild-type p53 but not by any of the p53 mutants (Figure 3a). These results are consistent with the observation that wild-type p53, but not mutant p53(R249S), induces Dkk-1 (Figure 1a). In contrast, the luciferase activity for mut-Dkk-1-Fluc was not increased by either wild-type p53 or p53 mutants (Figure 3b).

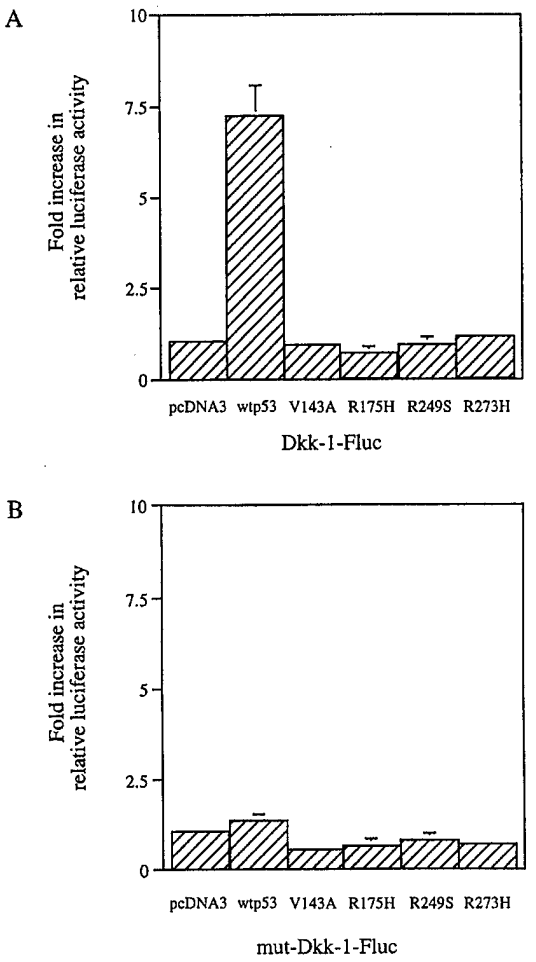


Figure 3 (**a**) The potential p53-binding site in the Dkk-1 gene is responsible to wild-type p53, but not p53 mutants *in vivo*. The 32-bp DNA fragment described in Figure 2 was cloned upstream of a minimal c-*fos* promoter and a firefly luciferase reporter gene (Johansen and Prywes, 1994), and the resulting construct designated Dkk-1-Fluc. Two μg of Dkk-1-Fluc was co-transfected into H1299 cells with 5 μg of pcDNA3 control vector or a vector that expresses wild-type p53, p53(V143A), p53(R175H), p53(R249S), or p53(R273H). Renilla luciferase assay vector pRL-CMV was also co-transfected as an internal control. Dual luciferase assay was performed according to the manufacturer's instruction (Promega, Madison, WI, USA). The fold increase in relative luciferase activity is a product of the luciferase activity induced by p53 divided by that induced by pcDNA3. (**b**) The mutated potential p53-binding site in the Dkk-1 gene is not responsive to either wild-type p53 or p53 mutants. A mutant version of the above 32-bp DNA fragment was made with the following sequence: 5′-AGCTTAGCaAAtCTTTAATGAA-CaAAtTTCA-3′ (top strand) and 5′-GATCTGAAaTTtGTTCAT-TAAAAGaTTtGCTA-3′ (bottom strand). The substituted nucleotides are shown in lower case. The mutant fragment was then cloned upstream of a minimal c-*fos* promoter and a firefly luciferase reporter gene, and the resulting construct designated mut-Dkk-1-Fluc. Luciferase assays with mut-Dkk-1-Fluc were performed as in (**a**)

Activation of p53 leads to at least two well-characterized cellular responses: cell cycle arrest and apoptosis (Chen, 1999; Ko and Prives, 1996; Levine, 1997). We wanted to determine whether Dkk-1, as a cellular target of p53, mediates p53 tumor suppression. To test this possibility, Dkk-1 was inducibly expressed in H1299 lung carcinoma and U118 glioma cells under a Tet-Off-tetracycline-regulated promoter. H1299 is p53-null but U118 harbors a p53 gene that can be activated by DNA damage (data not shown). Western blots from representative H1299 cell lines and U118 cell lines showed that, when induced, Dkk-1 was expressed with an apparent molecular mass of 29–35 kDa (data not shown). This range is consistent with previous reports of a slower migrating form of Dkk-1 that is N-linked glycosylated (Fedi *et al.*, 1999; Glinka *et al.*, 1998). We then measured the growth rates of these cells in the absence or presence of Dkk-1. We found that Dkk-1 has little, if any, effect on the growth rates of either H1299 or U118 cells (data not shown). In addition, no cell cycle arrest and apoptosis were detected using DNA histogram analysis and annexin V staining assay (data not shown). Thus, while Dkk-1 can suppress Wnt-induced transformation (Fedi *et al.*, 1999), it has no effect on the proliferation of cells that are not transformed by Wnt.

In this report we have demonstrated that Dkk-1 can be induced by p53 and DNA damage. We found a p53 responsive element located approximately 2100 nucleotides upstream of the Dkk-1 transcription start site, which may mediate DNA damage induction of Dkk-1. We also found that Dkk-1 has no effect on proliferation of cells that are not transformed by Wnt. Nevertheless, previous studies have shown that Dkk-1 is a potent antagonist of Wnt signaling necessary and sufficient for head induction in *Xenopus* (Glinka *et al.*, 1998) and that human Dkk-1 strongly suppresses Wnt-induced morphological transformation (Fedi *et al.*, 1999). Biochemical and genetic studies have shown that Dkk-1 antagonizes the Wnt signaling pathway, upstream of β-catenin and Dishevelled (Fedi *et al.*, 1999; Glinka *et al.*, 1998). Taken together, we propose that, by inducing Dkk-1, p53 plays an important role in suppressing Wnt-mediated tumor formation. Therefore, p53 dysfunction would alleviate the negative control of Wnt signaling by Dkk-1. As a result, uncontrolled Wnt signaling may be responsible for the early onset of mammary tumors in p53-null Wnt transgenic mice (Donehower *et al.*, 1995; Jones *et al.*, 1997).

Although the Wnt genes were initially identified as candidate proto-oncogenes, ectopic expression of Wnt induces axis duplication in *Xenopus* and Wnt gene deficiency prevents normal development of CNS, placenta, limbs, kidney, caudal somites and tailbud (Brown and Moon, 1998; Nusse and Varmus, 1992; Wodarz and Nusse, 1998). The negative control of Wnt signaling by Dkk-1 is also necessary for normal development (Glinka *et al.*, 1998). Interestingly, p53 induces Dkk-1 but p53 activity is not necessary for normal development in mice (Donehower *et al.*, 1992). This suggests that other factor(s) must be responsible for proper expression of Dkk-1 during development. In addition to the p53 responsive element, several potential regulatory elements, such as Sp1, MyoD, STAT, Oct-1/2, C/EBP-α/β, and GATA-1, -2 and -3, are found in the promoter region of the Dkk-1 gene (data not shown). Determining whether these transcription factors regulate Dkk-1 would lead to further our understanding of the role of Dkk-1 in development.

Acknowledgments

We would like to thank Rhea Markowitz for critical reading of this manuscript. This work is supported in part by National Cancer Institute Grant CA 76069 and the Department of Defense Army Breast Cancer Program DAMD17-97-1-7019.

References

Agarwal ML, Taylor WR, Chernov MV, Chernova OB and Stark GR. (1998). *J. Biol. Chem.*, **273**, 1–4.

Almog N and Rotter V. (1997). *Biochim. Biophys. Acta*, **1333**, F1–F27.

Bafico A, Gazit A, Pramila T, Finch PW, Yaniv A and Aaronson SA. (1999). *J. Biol. Chem.*, **274**, 16180–16187.

Brown JD and Moon RT. (1998). *Curr. Opin. Cell. Biol.*, **10**, 182–187.

Buckbinder L, Talbott R, Velasco-Miguel S, Takenaka I, Faha B, Seizinger BR and Kley N. (1995). *Nature*, **377**, 646–649.

Chen X. (1999). *Mol. Med. Today*, **5**, 387–392.

Chen X, Ko LJ, Jayaraman L and Prives C. (1996). *Genes Dev.*, **10**, 2438–2451.

Cook D, Fry MJ, Hughes K, Sumathipala R, Woodgett JR and Dale TC. (1996). *EMBO J.*, **15**, 4526–4536.

Donehower LA, Godley LA, Aldaz CM, Pyle R, Shi YP, Pinkel D, Gray J, Bradley A, Medina D and Varmus HE. (1995). *Genes Dev.*, **9**, 882–895.

Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery Jr CA, Butel JS and Bradley A. (1992). *Nature*, **356**, 215–221.

el-Deiry WS. (1998). *Semin. Cancer Biol.*, **8**, 345–357.

el-Deiry WS, Kern SE, Pietenpol JA, Kinzler KW and Vogelstein B. (1992). *Nat. Genet.*, **1**, 45–49.

el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW and Vogelstein B. (1993). *Cell*, **75**, 817–825.

Fedi P, Bafico A, Nieto Soria A, Burgess WH, Miki T, Bottaro DP, Kraus MH and Aaronson SA. (1999). *J. Biol. Chem.*, **274**, 19465–19472.

Finch PW, He X, Kelley MJ, Uren A, Schaudies RP, Popescu NC, Rudikoff S, Aaronson SA, Varmus HE and Rubin JS. (1997). *Proc. Natl. Acad. Sci. USA*, **94**, 6770–6775.

Friedlander P, Haupt Y, Prives C and Oren M. (1996). *Mol. Cell. Biol.*, **16**, 4961–4971.

Funk WD, Pak DT, Karas RH, Wright WE and Shay JW. (1992). *Mol. Cell. Biol.*, **12**, 2866–2871.

Giaccia AJ and Kastan MB. (1998). *Genes Dev.*, **12**, 2973–2983.

Glinka A, Wu W, Delius H, Monaghan AP, Blumenstock C and Niehrs C. (1998). *Nature*, **391**, 357–362.

Glinka A, Wu W, Onichtchouk D, Blumenstock C and Niehrs C. (1997). *Nature*, **389**, 517–519.

Hermeking H, Lengauer C, Polyak K, He T-C, Zhang L, Thiagalingam S, Kinzler KW and Vogelstein B. (1997). *Mol. Cell.*, **1**, 3–11.

Howes KA, Ransom N, Papermaster DS, Lasudry JG, Albert DM and Windle JJ. (1994). *Genes Dev.*, **8**, 1300–1310.

Hsieh JC, Kodjabachian L, Rebbert ML, Rattner A, Smallwood PM, Samos CH, Nusse R, Dawid IB and Nathans J. (1999). *Nature,* **398,** 431–436.

Hundley JE, Koester SK, Troyer DA, Hilsenbeck SG, Subler MA and Windle JJ. (1997). *Mol. Cell. Biol.,* **17,** 723–731.

Johansen FE and Prywes R. (1994). *Mol. Cell. Biol.,* **14,** 5920–5928.

Jones JM, Attardi L, Godley LA, Laucirica R, Medina D, Jacks T, Varmus HE and Donehower LA. (1997). *Cell Growth Differ.,* **8,** 829–838.

Kastan MB, Zhan Q, el-Deiry WS, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B and Fornace Jr AJ. (1992). *Cell.* **71,** 587–597.

Kern SE, Kinzler KW, Bruskin A, Jarosz D, Friedman P, Prives C and Vogelstein B. (1991). *Science,* **252,** 1708–1711.

Klingensmith J, Nusse R and Perrimon N. (1994). *Genes Dev.,* **8,** 118–130.

Ko LJ and Prives C. (1996). *Genes Dev.,* **10,** 1054–1072.

Krasnow RE, Wong LL and Adler PN. (1995). *Development,* **121,** 4095–4102.

Levine AJ. (1997). *Cell,* **88,** 323–331.

Leyns L, Bouwmeester T, Kim SH, Piccolo S and De Robertis EM. (1997). *Cell,* **88,** 747–756.

Miyashita T, Krajewski S, Krajewska M, Wang HG, Lin HK, Liebermann DA, Hoffman B and Reed JC. (1994). *Oncogene,* **9,** 1799–1805.

Munemitsu S, Albert I, Rubinfeld B and Polakis P. (1996). *Mol. Cell. Biol.,* **16,** 4088–4094.

Nelson WG and Kastan MB. (1994). *Mol. Cell. Biol.,* **14,** 1815–1823.

Nusse R and Varmus HE. (1992). *Cell,* **69,** 1073–1087.

Pai LM, Orsulic S, Bejsovec A and Peifer M. (1997). *Development,* **124,** 2255–2266.

Pan H and Griep AE. (1995). *Genes Dev.,* **9,** 2157–2169.

Piccolo S, Agius E, Leyns L, Bhattacharyya S, Grunz H, Bouwmeester T and De Robertis EM. (1999). *Nature,* **397,** 707–710.

Polyak K, Xia Y, Zweier JL, Kinzler KW and Vogelstein B. (1997). *Nature,* **389,** 300–305.

Rouault JP, Falette N, Guehenneux F, Guillot C, Rimokh R, Wang Q, Berthet C, Moyret-Lalle C, Savatier P, Pain B, Shaw P, Berger R, Samarut J, Magaud JP, Ozturk M, Samarut C and Puisieux A. (1996). *Nat. Genet.,* **14,** 482–486.

Symonds H, Krall L, Remington L, Saenz-Robles M, Lowe S, Jacks T and Van Dyke T. (1994). *Cell,* **78,** 703–711.

Theisen H, Purcell J, Bennett M, Kansagara D, Syed A and Marsh JL. (1994). *Development,* **120,** 347–360.

Wang S, Krinks M, Lin K, Luyten FP and Moos Jr M. (1997). *Cell,* **88,** 757–766.

Wang XW, Zhan Q, Coursen JD, Khan MA, Kontny HU, Yu L, Hollander MC, O'Connor PM, Fornace Jr AJ and Harris CC. (1999). *Proc. Natl. Acad. Sci. USA,* **96,** 3706–3711.

Wodarz A and Nusse R. (1998). *Annu. Rev. Cell. Dev. Biol.,* **14,** 59–88.

Wong LL and Adler PN. (1993). *J. Cell. Biol.,* **123,** 209–221.

Wu GS, Burns TF, McDonald III ER, Jiang W, Meng R, Krantz ID, Kao G, Gan DD, Zhou JY, Muschel R, Hamilton SR, Spinner NB, Markowitz S, Wu G and el-Deiry WS. (1997). *Nat. Genet.,* **17,** 141–143.

Wu X, Bayle JH, Olson D and Levine AJ. (1993). *Genes Dev.,* **7,** 1126–1132.

Yin Y, Terauchi Y, Solomon GG, Aizawa S, Rangarajan PN, Yazaki Y, Kadowaki T and Barrett JC. (1998). *Nature,* **391,** 707–710.

Yost C, Torres M, Miller JR, Huang E, Kimelman D and Moon RT. (1996). *Genes Dev.,* **10,** 1443–1454.

Zhu J, Zhou W, Jian J and Chen X. (1998). *J. Biol. Chem.,* **273,** 13030–13036.

Zhu KJW, Zhu J, Jiang J, Shou J and Chen X. (1999). *Oncogene,* **18,** 7740–7747.

Definition of the p53 functional domains necessary for inducing apoptosis

*Jianhui Zhu, Shunzhen Zhang, Jieyuan Jiang, and Xinbin Chen**

Institute of Molecular Medicine and Genetics

Medical College of Georgia, Augusta, GA 30912

*Corresponding author.

Dr. Xinbin Chen

CB-2803

Institute of Molecular Medicine and Genetics

Medical College of Georgia

Augusta, GA 30912

Phone (706) 721-8760

Fax (706) 721-8752

Email xchen@mail.mcg.edu

Running Title: p53 functional domains for apoptosis

Summary

The p53 protein contains several functional domains necessary for inducing cell cycle arrest and apoptosis. The C-terminal basic domain, within residues 364-393, and the proline-rich domain, within residues 64-91, are required for apoptotic activity. In addition, activation domain 2, within residues 43-63, is necessary for apoptotic activity when the N-terminal activation domain 1, within residues 1-42, is deleted (ΔAD1) or mutated ($AD1^-$). Here we found that an activation domain 2 mutation at residues 53-54 ($AD2^-$) abrogates the apoptotic activity but has no significant effect on cell cycle arrest. We also found that p53(ΔAD2), which lacks activation domain 2, are inert in inducing apoptosis. p53($AD2^-$ΔBD), which is defective in activation domain 2 and lacks the C-terminal basic domain, p53(ΔAD2ΔBD), which lacks both activation domain 2 and the C-terminal basic domain, and p53(ΔPRDΔBD), which lacks both the proline-rich domain and the C-terminal basic domain, are also inert in inducing apoptosis. All four mutants are still active in inducing cell cycle arrest, albeit to a lesser extent than wild-type p53. Interestingly, we found that deletion of the N-terminal activation domain 1 alleviates the requirement of the C-terminal basic domain for apoptotic activity. Thus, we have generated a small but potent p53(ΔAD1ΔBD) molecule. Furthermore, we found that at least two of the three domains, that is, activation domain 1, activation domain 2 and the proline-rich domain, are required for inducing cell cycle arrest. Taken together, our results suggest that activation domain 2 and the proline-rich domain form an activation domain for inducing pro-apoptotic genes or inhibiting anti-apoptotic genes. The C-terminal basic domain is required for maintaining this activation domain competent for transactivation or transrepression.

Introduction

Activation of p53 leads to at least two well-defined cellular responses: cell cycle arrest and apoptosis (1-4). Based on these activities and other characteristics (1,5), the p53 protein can be divided into several functional domains. These are activation domain 1, within residues 1-42 (6-8), activation domain 2, within residues 43-63 (9-11), the proline-rich domain, within residues 64-91 (12), the sequence-specific DNA-binding domain, within residues 100-300 (1), the nuclear localization signal, within residues 316-325 (13), the tetramerization domain, within residues 334-356 (14) and which also contains a nuclear export signal (15), and the C-terminal basic domain, within residues 364-393 (1,5).

p53 is frequently mutated in cancers. Mutations in the p53 DNA binding domain, or certain mutations in the nuclear localization signal and tetramerization domain that indirectly affect DNA binding, abrogate or diminish p53 activity in cell cycle arrest and apoptosis (1,5). The proline-rich domain has been shown to be required for efficient growth suppression (12). Recent experiments indicate that the proline-rich domain is necessary for apoptosis but not cell cycle arrest (16-18). In addition, the proline-rich domain plays an important role in the induction of several endogenous target genes, but is not required for activation of the exogenously introduced promoters of these target genes (17). These results suggest that the proline-rich domain may participate in the induction of cellular target gene(s) responsible for mediating apoptosis. However, the role of other p53 functional domains, especially the N-terminal activation domain 1 and the C-terminal basic domain, in apoptosis is still not certain. Earlier reports have shown that in some experimental protocols (19-21), including our own (22), p53 transactivation activity is dispensable for apoptosis. It should be noted that this conclusion is based at least in part on the observation that an activation domain 1 deficient mutant (a double point mutation at residues 22-23; $AD1^-$) is capable of inducing apoptosis (21,22). Recently, we and others have shown that p53($AD1^-$) contains an intact activation domain 2 (9-11), and therefore, p53($AD1^-$) is still competent in transactivation (10). Furthermore, when both activation domain 1 and activation domain 2 are mutated (a quadruple point mutation at residues 22-23 and 53-54; $AD1^-AD2^-$), the resulting

protein is inert in transactivation and in inducing cell cycle arrest and apoptosis (9-11).

The C-terminal basic domain has been subjected to extensive analysis and all evidence suggests that the basic domain is a regulatory domain. This basic domain can regulate the DNA binding activity when it is phosphorylated (1,5), acetylated (23-25), deleted (26), or associated with anti-p53 antibody (26,27) or peptides derived from the C-terminus of p53 (28,29). Interestingly, the mechanism by which these latter peptides enhance p53 DNA binding activity is the ability of the peptides to interact with three separate domains in p53, that is, the proline-rich domain (30), the DNA binding domain (31), and the C-terminal basic domain (30,31). The C-terminal basic domain also interacts with several cellular proteins, such as TFIIH subunits XPB and XPD (32,33), and Werner syndrome protein (WRN) (34,35), which all lead to efficient induction of p53-mediated apoptosis. These results support a hypothesis that the C-terminal basic domain is a negative regulatory domain whose effect on the DNA binding activity can be alleviated by interacting with other cellular proteins, peptides derived from the p53 C-terminus, or other modifications. However, several groups have shown that p53(ΔBD), which lacks the C-terminal basic domain, has a reduced ability to induce several cellular target genes and becomes incapable of inducing apoptosis (22,32,36). These results suggest that the C-terminal basic domain can regulate p53 activity both positively and negatively.

In this study, we show that activation domain 2 and the proline-rich domain form an activation domain for inducing pro-apoptotic genes or inhibiting anti-apoptotic genes. The C-terminal basic domain is required for maintaining this activation domain competent for transactivation or transrepression. We also found that an activation domain capable of inducing at least partial cell cycle arrest can be formed by activation domain 1 plus activation domain 2, activation domain 1 plus the proline-rich domain, or activation domain 2 plus the proline-rich domain. The ability of these activation domains to induce cell cycle arrest can be enhanced by the presence of the C-terminal basic domain.

Experimental procedures

Plasmids and mutagenesis. Mutant p53 cDNA constructs were generated by PCR and mutations were confirmed by DNA sequencing. All p53 proteins were tagged at their N-termini with an influenza hemagglutinin (HA) peptide recognizable by anti-HA antibody 12CA5. HA-tagged wild-type p53 was generated using 5' end primer 5HA, GAT CGA ATT CAC CAT GGG CTA CCC ATA CGA TGT TCC AGA TTA CGC TGA GGA GCC GCA GTC AGA TCC, and 3' end primer C393, GAT CGA ATT CTC AGT CTG AGT CAG GCC CTT. To generate p53(AD2$^-$), cDNA fragments encoding amino acids 1-59 and 60-393 were amplified independently and ligated through an internal Ava II site. The cDNA fragment encoding amino acids 1-59 was amplified by 5' end primer 5HA and 3' end primer C59, TTC ATC TGG ACC TGG GTC TTC AGT GCT CTG TTG TTC AAT ATC. The cDNA fragment encoding amino acids 60-393 was amplified by 5'end primer N60, ACT GAA GAC CCA GGT CCA, and 3' end primer C393. To generate p53(ΔAD2), a cDNA fragment that encodes residues 41 to 393 but lacks residues 43-63 was amplified by 5' end primer AD2, TTG CAA TGG ATG ATG CTC CCA GAA TGC CAG A, and 3' end primer C393. This fragment was then used to replace the HA-tagged wild-type p53 from residues 41-393 at a Bsr D1 site. To generate p53(ΔAD2ΔPRD), a cDNA fragment that encodes residues 41 to 393 but lacks residues 43-91 was amplified by 5' end primer AP5, TTG CAA TGG ATG ATC CCC TGT CGT CTT CTG T, and 3' end primer C393. This fragment was then used to replace the HA-tagged wild-type p53 from residues 41-393 at a Bsr D1 site. p53(ΔAD1), p53(ΔPRD), p53(ΔBD), p53(ΔAD1AD2$^-$), and p53(ΔAD1ΔAD2) were generated as described previously (10,17,22). To generate p53(ΔAD1ΔPRD), p53(ΔPRD) cDNA was amplified by 5' end primer N43, GAT CGA ATT CAC CAT GGG CTA CCC ATA CGA TGT TCC AGA TTA CGC TTT GAT GCT GTC CCC G, and 3' end primer C393. To generate p53(AD2$^-$ΔBD), p53(ΔAD2ΔBD), p53(ΔPRDΔBD), p53(ΔAD1ΔBD), p53(ΔAD1AD2$^-$ΔBD), p53(ΔAD1ΔPRDΔBD), p53(ΔAD1ΔAD2ΔBD), and p53(ΔAD2ΔPRDΔBD), the 3' cDNA fragments starting from the Stu I site in p53(AD2$^-$), p53(ΔAD2), p53(ΔPRD), p53(ΔAD1), p53(ΔAD1AD2$^-$), p53(ΔAD1ΔPRD), p53(ΔAD1ΔAD2), and p53(ΔAD2ΔPRD) were replaced

with the corresponding cDNA fragment in p53(ΔBD).

The above mutant p53 cDNAs were cloned separately into a tetracycline-regulated expression vector, pUHD10-3, at its Eco RI site (37) and the resulting plasmids were used to generate cell lines that inducibly express p53.

Cell lines. H1299 and MCF7 cell lines that express inducible proteins of interest were generated as previously described (10,17,22). The H1299 cell lines p53-3, p53(R249S)-4, p53(AD1$^-$)-2, p53(ΔBD)-1, p53(ΔPRD)-5, and p53(ΔAD1)-2 were as previously described (10,17,22).

Western blot analysis. Western blot analysis was performed as described (10,17,22), with anti-p53 monoclonal antibody Pab240, anti-HA monoclonal antibody12CA5 (Boehringer Mannhein Biochemicals, Indianapolis, IN), anti-actin polyclonal antibody (Sigma), and anti-p21 monoclonal antibody (Ab-1) (Oncogene Research Products, Cambridge, MA).

Growth rate analysis, trypan blue dye exclusion assay, DNA histogram analysis and annexin V staining. Growth rate analysis, trypan blue dye exclusion assay, and DNA histogram analysis were performed as described previously (10,17,22). Propidium iodide and RNase A were purchased from Sigma (St. Louis, MO). Fluorescein isothiocyanate (FITC)-labeled annexin V was purchased from Boehringer Mannhein Biochemicals (Indianapolis, IN) and staining was performed as described by the manufacturer.

RNA isolation and Northern blot analysis. Total RNA was isolated using Trizol reagents (BRL-GIBCO). Northern blot analysis was performed as described (10). The *p21*, *BAX*, and *GADPH* probes were prepared as previously described (10).

Results

The activity of activation domain 2 is necessary for inducing apoptosis. Previously, we have shown that the activity of activation domain 2 is required for inducing apoptosis when a double point mutation at residues 22-23 or deletion of the N-terminal 42 amino acid residues renders activation domain 1 dysfunctional (10). To further determine the function of activation domain 2 in apoptosis, we generated an activation domain 2 deficient mutant, p53(AD2$^-$), which contains a double point mutation at residues 53-54. We then established several cell lines that inducibly express this mutant in p53-null H1299 lung carcinoma cells. Western blots from two representative cell lines, p53(AD2$^-$)-6 and -8, are shown in Fig. 1A. After normalization to the levels of actin protein expressed, we found that the levels of p53 protein in p53(AD2$^-$)-6 and -8 cells were comparable to that in p53-3 and HA-p53-15 cells, which express wild-type p53 and HA-tagged wild-type p53, respectively (Fig. 1A, upper two panels, compare lanes 5-8 with lanes 1-4). To determine the transcriptional activity of p53(AD2$^-$), we measured the level of p21 protein induced by p53(AD2$^-$). Surprisingly, we found that the ability of p53(AD2$^-$) to induce p21 was severely diminished (Fig. 1A, p21 panel, lanes 5-8). These results are similar to that observed for the activation domain 1 deficient mutant (6,10,22). In contrast, p21 was strongly induced by wild-type p53 and HA-tagged wild-type p53 (Fig. 1A, p21 panel, lanes 1-4).

One of the hallmarks for p53 when overexpressed in cells is growth suppression (1-3). The HA-tagged wild-type p53 protein in HA-p53-15 cells, like the untagged wild-type p53 in p53-3 cells (10,22), inhibits cell proliferation (data not shown). To determine the activity of p53(AD2$^-$) in H1299 cells, the growth rate of p53(AD2$^-$)-6 cells was determined over a 5-day period. When induced to express p53(AD2$^-$), cells failed to multiply (Fig. 1B), but visible microscopic cell death was not significantly increased (data not shown).

Previously, several studies have shown that the C-terminal basic domain is necessary for inducing apoptosis but not cell cycle arrest (22,32). To determine whether this domain has any effect on the ability of p53(AD2$^-$) to induce growth suppression, we generated p53(AD2$^-\Delta$BD),

which is deficient in activation domain 2 and has a deletion of the C-terminal basic domain. We then established several cell lines that inducibly express p53(AD2$^-$ΔBD). Western blots from three representative cell lines, p53(AD2$^-$ΔBD)-2, -8, and -9, are shown in Fig. 1A. We found that the levels of p53 in these cells were comparable to that in HA-p53-15 and p53(ΔBD)-1 cells (Fig. 1A, upper two panels, compare lanes 3, 4, and 9-16). p53(ΔBD)-1 cells are derived from H1299 cells that inducibly express p53(ΔBD), which lacks the C-terminal basic domain (22). Similarly, the transcriptional activity of p53(AD2$^-$ΔBD) was determined by measuring the level of p21 induced. We found that, like p53(AD2$^-$), the ability of p53(AD2$^-$ΔBD) to induce p21 was significantly diminished (Fig. 1A, p21 panel, compare lanes 11-16 with lanes 1-4). In contrast, p21 was strongly induced by p53(ΔBD) (Fig. 1A, p21 panel, lanes 9-10), consistent with previous reports (22,32). Nevertheless, growth rate analysis showed that p53(AD2$^-$ΔBD) was still capable of inhibiting cell growth (Fig. 1C), albeit to a lesser extent than p53(AD2$^-$) (Fig. 1B).

To determine whether the growth suppression by p53(AD2$^-$) is due to cell cycle arrest, apoptosis and/or both, we performed a DNA histogram analysis and an annexin V staining assay. When induced to express the mutant p53(AD2$^-$) for two days, we found that the percentage of cells in S phase decreased from 35 to 8% while cells in G1 increased from 49 to 75%, suggesting that p53(AD2$^-$) arrested cells primarily in G1 (Fig. 1D-E). However, no apparent apoptosis was detected by either DNA histogram analysis (Fig. 1D-E) or annexin V staining (Fig. 1F-G). Thus, the activity in activation domain 2 is necessary for inducing apoptosis. As a positive control, we analyzed p53-3 and HA-p53-15 cells. When induced to express wild-type or HA-tagged p53 for two days, we found that both p53-producing cells were arrested primarily in G1 and underwent apoptosis, consistent with previous reports (10,22). We also analyzed p53(AD2$^-$ΔBD)-9 cells. We found that no significant apoptosis was observed and cells primarily arrested in G1 when induced to express p53(AD2$^-$ΔBD) (data not shown).

To determine the activity of the entire activation domain 2 (residues 43-62), we generated p53(ΔAD2), which lacks the entire activation domain 2 and p53(ΔAD2ΔBD), which lacks activation domain 2 and the C-terminal basic domain. We then established several cell lines that

inducibly express p53(ΔAD2) and p53(ΔAD2ΔBD), respectively (Fig. 2A and 2C). We found that p53(ΔAD2) and p53(ΔAD2ΔBD) suppressed cell proliferation (Fig. 2B and 2D), albeit to a lesser extent than p53(AD2$^-$) and p53(AD2$^-$ΔBD) (Fig. 1B and 1C). Furthermore, we found that cells were arrested primarily in G1 but did not undergo apoptosis when induced to express these p53 mutants (data not shown; also see Table 1). However, we found that p21 was not significantly induced (Fig. 2A and 2C), suggesting that p53-dependent cell cycle arrest in G1 can be mediated by a gene(s) other than p21.

The proline-rich domain contributes to the ability of p53 to induce cell cycle arrest. Previously, we and others have shown that the proline-rich domain (16-18) and the C-terminal basic domain (22,32) are necessary for inducing apoptosis but not cell cycle arrest. To determine whether both domains are dispensable for inducing cell cycle arrest, we generated p53(ΔPRDΔBD), which lacks both the proline-rich domain and the C-terminal basic domain. We then established several cell lines that inducibly express this mutant. Western blots from three representative cell lines, p53(ΔPRDΔBD)-2, -6, and -7, are shown in Fig. 2E. We found that the level of p53 expressed in p53(ΔPRDΔBD)-2 cells was comparable to that in p53-3, HA-p53-15, and p53(ΔBD)-1, but slightly lower than that in p53(ΔPRD)-5, which inducibly expresses a p53 mutant lacking the proline-rich domain (Fig. 2E, p53 panel). To determine whether p21 can be induced, we found that p53(ΔPRDΔBD) was much less potent in inducing p21 than wild-type p53, HA-tagged p53, p53(ΔBD), or p53(ΔPRD) (Fig. 2E, p21 panel). However, when the DNA binding activity was determined *in vitro*, we found that p53(ΔPRDΔBD) was as potent as wild-type p53 in binding to the ribosomal gene cluster p53 response element (data not shown). This suggests that deletion of both the proline-rich domain and the C-terminal basic domain does not affect the activity of the p53 DNA binding domain. Growth rate analysis showed that p53(ΔPRDΔBD) had a much reduced ability to suppress cell proliferation (Fig. 2F). In addition, DNA histogram analysis and annexin V staining assay showed that a partial arrest in G1, but no apoptosis, was detected in p53(ΔPRDΔBD)-2 cells (data not shown).

p53(ΔAD1ΔBD) is small but potent in inducing cell cycle arrest and apoptosis. We

and others have shown that p53(ΔBD), which lacks the C-terminal basic domain, is inactive in inducing apoptosis (22,32,36) whereas p53(ΔAD1), which lacks activation domain 1 (residues 1-42), is very active (10). To determine whether the C-terminal basic domain is necessary for p53(ΔAD1) to induce apoptosis, we generated p53(ΔAD1ΔBD), which lacks activation domain 1 and the C-terminal basic domain. We then established several cell lines that inducibly express p53(ΔAD1ΔBD). Western blots from three representative cell lines, p53(ΔAD1ΔBD)-3, -6, and -7, are shown in Fig. 3A. We found that the level of p53 expressed in these cells was comparable to that in p53-3, HA-p53-15, and p53(ΔBD)-1 cells, but lower than that in p53(ΔAD1)-2 cells (Fig. 3A, p53 panel). p53(ΔAD1)-2 cells are derived from H1299 cells that inducibly express p53(ΔAD1), which lacks activation domain 1 (10). We found that p53(ΔAD1ΔBD) retained the ability to induce p21. Induction of p21 by p53(ΔAD1ΔBD) was greater than induction by p53(ΔAD1) but less than induction by wild-type p53 and p53(ΔBD) (Fig. 3A, p21 panel). Growth rate analysis showed that cells failed to multiply, detached from plates, and shrank to form apoptotic bodies when induced to express p53(ΔAD1ΔBD) (Fig. 3B and 3C). DNA histogram analysis showed that the percentage of cells in S phase decreased from 35 to 11% but the percentage of cells in G1 increased from 55 to 75%, suggesting that these cells arrested primarily in G1 (Fig. 3D-E). We also found that the number of cells with a sub-G1 DNA content was not significantly increased. However, when stained for annexin V, we found that the percentage of stained cells increased from 7 to 31%, suggesting that these cells also underwent apoptosis (Fig. 3F-G).

To further confirm the ability of p53(ΔAD1ΔBD) to induce apoptosis, we generated several MCF7 breast carcinoma cell lines that inducibly express wild-type p53 and p53(ΔAD1ΔBD). Western blots from one representative cell line that inducibly expresses wild-type p53 (MCF7-p53-24) and two that inducibly express p53(ΔAD1ΔBD) (MCF7-p53(ΔAD1ΔBD)-7 and –15) are shown in Fig. 4A. We found that the level of p53 induced in MCF7-p53(ΔAD1ΔBD)-7 and –15 cells was slightly lower than in MCF7-p53-24 cells (Fig. 4A, p53 panel). When the level of p21 was measured to determine the transcriptional activity of

p53(ΔAD1ΔBD), we found that p53(ΔAD1ΔBD) was potent in transactivation (Fig. 4A, p21 panel). This result is similar to that obtained in H1299 cells (Fig. 3A). Growth rate analysis showed that cells failed to multiply, detached from plates, and shrank to form apoptotic bodies when induced to express wild-type p53 or p53(ΔAD1ΔBD) (Fig. 4B-C). DNA histogram analysis showed that the percentage of cells that had a sub-G1 DNA content was increased from 3 to 37% by wild-type p53 (Fig. 4D-E) and from 4 to 49% by p53(ΔAD1ΔBD) (Fig. 4H-I). In addition, annexin V staining assay showed that the percentage of the annexin V-stained cells was increased from 7 to 28% by wild-type p53 and from 9 to 29% by p53(ΔAD1ΔBD). These data indicate that p53(ΔAD1ΔBD) is a potent apoptotic inducer.

At least two of the three domains, that is, activation domain 1, activation domain 2, and the proline-rich domain, are required for inducing cell cycle arrest. To further define the role of activation domain 1, activation domain 2, the proline-rich domain, and the C-terminal basic domain in inducing cell cycle arrest and apoptosis, we generated six p53 mutants that are dysfunctional in two or three of the four functional domains (Fig. 5). We then established several cell lines that inducibly express these p53 mutants individually (Fig. 5). These are p53(ΔAD1AD2ΔBD) (Fig. 5A), p53(ΔAD1ΔAD2ΔBD) (Fig. 5B), p53(ΔAD1ΔPRDΔBD) (Fig. 5C), p53(ΔAD1ΔPRD) (Fig. 5D), p53(ΔAD2ΔPRD) (Fig. 5E), and p53(ΔAD2ΔPRDΔBD) (Fig. 5E). The level of p53 expressed in some of these mutant p53-producing cells was comparable to, or higher than, that in p53-3 cells (Fig. 5A-E, p53 panel). However, none of these mutants were capable of inducing p21 (Fig. 5A-E, p21 panel). In addition, cell cycle arrest and apoptosis were not detected by growth rate and DNA histogram analyses and annexin V staining assay (data not shown). These data suggest that at least two of the three domains (activation domain 1, activation domain 2, and the proline-rich domain) are required for p53 activity.

Regulation of *p21* and *BAX* by p53 mutants. To determine the ability of various p53 mutants that lack activation domain 1, activation domain 2, and/or the C-terminal basic domain in inducing *p21* and *BAX*, we performed a Northern blot analysis (Fig. 6). We found that wild-type p53 was very active (lanes 1-2). p53(R249S), a tumor-derived mutant that is defective in

the DNA binding domain, was nearly inert (lanes 3-4). Although deletion of the C-terminal basic domain renders p53 constitutively active in binding to DNA in vitro (26), the ability of p53(ΔBD) to induce *p21* and *BAX* was significantly reduced as compared to that of wild-type p53 (compare lanes 1-2 and 7-8). p53(AD1$^-$) (lanes 5-6), p53(ΔAD1) (lanes 9-10), p53(AD2$^-$) (lanes 13-14), and p53(AD2$^-$ΔBD) (lanes 15-16) were extremely weak in inducing *p21* and *BAX* (2 fold or less). It should be mentioned that p53(AD2$^-$) is extremely potent in inducing G1 arrest (Fig. 1D-E), suggesting that a gene(s) other than p21 is responsible for this. Furthermore, when acitvation domain 1 and the basic domain were deleted, the ability of p53(ΔAD1ΔBD) to induce *p21* and *BAX* was partially restored (lanes 11-12), consistent with the result detected by Western blot analysis (Fig. 3A).

Discussion

p53 induces apoptosis but the underlying mechanism remains unclear. In order to determine this mechanism, two major questions need to be addressed. What domains in p53 are required? Is p53 transcriptional activity necessary for inducing apoptosis? Previous attempts to answer these questions have been inconclusive, since different experimental systems have been used (1,2). These include various types of cell lines and methods to express p53 (transient versus stable; ectopic versus inducible) and different types of p53 mutants (temperature-sensitive mutant versus wild-type p53; point mutations versus deletion mutations). To avoid these problems, we have applied the tetracycline inducible expression system to stably express various p53 mutants in p53-null H1299 cells. On the basis of the results obtained in this study, summarized in Table 1, and several previous studies (11,12,16,32,36,38-40), including our own (10,17,22), we propose the following model for p53 functional domains in apoptosis (Fig. 7). First, p53 DNA binding activity is necessary for apoptosis since mutants that are defective in the DNA binding and tetramerization domains are inert. Second, activation domain 2 and the proline-rich domain can form an activation domain for transactivating pro-apoptotic genes or transrepressing anti-apoptotic genes, since mutation or deletion in either one of the domains abrogates the apoptotic activity. Third, activation domain 1 is not required since deletion of or mutation in activation domain 1 (p53(ΔAD1); p53(AD1$^-$)) has little effect on apoptosis. Fourth, the C-terminal basic domain is necessary for maintaining p53 competent in inducing apoptosis, probably by relieving the inhibitory activity of activation domain 1, since p53(ΔAD1ΔBD), but not p53(ΔBD), is capable of inducing apoptosis.

Several p53 inducible genes, such as *BAX* (41), *KILLER/DR5* (42), and several *PIGs* (43), may participate in the apoptotic process. These genes can be induced by either p53(ΔPRD) (17) or p53(AD2$^-$) (data not shown), both of which are active in inducing cell cycle arrest but not apoptosis, suggesting that these genes are not required or insufficient for inducing apoptosis. Recent evidence has shown that p53 can repress specific genes, such as MAP4 (44). It is possible that transrepression of anti-apoptotic genes plays an important role in p53-mediated

apoptosis. Therefore, the cell lines that inducibly express the p53 mutants described in this study, especially p53(ΔAD1ΔBD), can be used to identify and determine whether a cellular gene is necessary for mediating p53-dependent apoptosis.

p53 transcriptional activity has been shown to be necessary for inducing cell cycle arrest (1,2,4,45). In this study, we extend this observation. We found that an activation domain capable of inducing at least partial cell cycle arrest can be formed by activation domain 1 plus activation domain 2, activation domain 1 plus the proline-rich domain, or activation domain 2 plus the proline-rich domain (Table 1). When two of the three domains, i.e., activation domain 1, activation domain 2, and the proline-rich domain, become dysfunctional, the activity in cell cycle arrest is abrogated (Table 1). It should be mentioned that p53(AD1$^-$) is defective in inducing cell cycle arrest although two functional domians, that is, activation domain 2 and the proline-rich domain, are still intact (22). However, when part or all of the residues for activation domain 1 are deleted, as in p53(Δ1-23) and p53(ΔAD1), the ability to induce cell cycle arrest is retained. This suggests that the presence of the mutated activation domain 1 may mask the activity of, or inhibit the interaction of a potential co-activator (or an adaptor) with, the activation domain formed by activation domain 2 and the proline-rich domain necessary for transactivation or transrepression.

The search for mediators of p53-dependent cell cycle arrest has identified many cellular p53 target genes (1,4,46). $p21^{Cip1/Waf1}$, a well characterized cyclin-dependent kinase inhibitor, can mediate cell cycle arrest in G1 when overexpressed (22,47-51). Previous studies have shown that p53(AD1$^-$), which is deficient in inducing p21, is incapable of inducing arrest in G1, consistent with the hypothesis that p21 plays an important role in mediating p53-dependent arrest in G1 (22,40). In this study, we found that p53(AD2$^-$) is extremely active in inducing arrest in G1, suggesting that activation domain 1, but not activation domain 2, plays an important role in inducing cell cycle arrest. However, p21 is only slightly induced by p53(AD2$^-$) (Fig. 1A). Since p53(AD1$^-$AD2$^-$), which is deficient in both activation domain 1 and activation domain 2, is inert in inducing cell cycle arrest (9-11), this suggests that a gene(s) responsible for arrest by

p53(AD2$^-$) must be induced. This is not surprising since DNA damage-induced G1 arrest is delayed but not abolished in p21-null fibroblasts from p21 deficient mice (52,53). Therefore, the cell line that inducibly expresses p53(AD2$^-$) can be used to identify other novel gene(s) responsible for G1 arrest.

Previously, several studies have shown that the p53 protein can be cleaved by cellular proteases in cells treated with DNA damaging agents, which leads to formation of several smaller polypeptides with molecular masses ranging from 35-50 kDa (54-58). In addition, the cleavage of p53 is concomitant with the onset of apoptosis in cells treated with DNA damaging agents, suggesting that the cleaved p53 polypeptides are potent in p53 activity and may participate in the apoptotic process (58). Interestingly, one of the cleaved p53 polypeptides, p50, is p53(ΔN23), which lacks the N-terminal 23 residues (58). We have shown previously that p53(ΔN23) is active in inducing cell cycle arrest and apoptosis (10). Thus, the cellular machinery can generate an active but smaller p53 polypeptide that would not be subject to negative regulation by MDM2 (59-63). It is not clear whether p53(ΔAD1ΔBD) is an *in vivo* cleavage product of p53. However, since p53(ΔAD1ΔBD) lacks the MDM2 binding site, it would not be subjected to the negative regulation by MDM2. Thus, p53(ΔAD1ΔBD) represents a small but potent, apoptosis-inducing form of p53. Recent clinical tries have shown that adenoviruses expressing p53 are effective in treating some advanced forms of human cancers (64,65). We suggest that p53(ΔAD1ΔBD) is a good candidate to replace the larger, unwieldy wild-type p53 in cancer gene therapy.

Acknowledgment

We would like to thank Rhea Markowitz, Tara Pipes, Susan Nozell, and Michael Dohn for critical reading of this manuscript.

This work is supported in part by National Cancer Institute Grant CA 76069 and the Department of Defense Army Breast Cancer Program DAMD17-97-1-7019.

References

1. Ko, L. J., and Prives, C. (1996) *Genes Dev* **10**(9), 1054-72

2. Levine, A. J. (1997) *Cell* **88**(3), 323-31

3. Agarwal, M. L., Taylor, W. R., Chernov, M. V., Chernova, O. B., and Stark, G. R. (1998) *J Biol Chem* **273**(1), 1-4

4. Chen, X. (1999) *Mol Med Today* **5**(9), 387-392

5. Giaccia, A. J., and Kastan, M. B. (1998) *Genes Dev* **12**(19), 2973-83

6. Lin, J., Chen, J., Elenbaas, B., and Levine, A. J. (1994) *Genes Dev* **8**(10), 1235-46

7. Unger, T., Mietz, J. A., Scheffner, M., Yee, C. L., and Howley, P. M. (1993) *Mol Cell Biol* **13**(9), 5186-94

8. Chang, J., Kim, D. H., Lee, S. W., Choi, K. Y., and Sung, Y. C. (1995) *J Biol Chem* **270**(42), 25014-9

9. Candau, R., Scolnick, D. M., Darpino, P., Ying, C. Y., Halazonetis, T. D., and Berger, S. L. (1997) *Oncogene* **15**(7), 807-16

10. Zhu, J., Zhou, W., Jiang, J., and Chen, X. (1998) *J Biol Chem* **273**(21), 13030-6

11. Venot, C., Maratrat, M., Sierra, V., Conseiller, E., and Debussche, L. (1999) *Oncogene* **18**(14), 2405-10

12. Walker, K. K., and Levine, A. J. (1996) *Proc Natl Acad Sci U S A* **93**(26), 15335-40

13. Shaulsky, G., Goldfinger, N., Tosky, M. S., Levine, A. J., and Rotter, V. (1991) *Oncogene* **6**(11), 2055-65

14. Sturzbecher, H. W., Brain, R., Addison, C., Rudge, K., Remm, M., Grimaldi, M., Keenan, E., and Jenkins, J. R. (1992) *Oncogene* **7**(8), 1513-23

15. Stommel, J. M., Marchenko, N. D., Jimenez, G. S., Moll, U. M., Hope, T. J., and Wahl, G. M. (1999) *Embo J* **18**(6), 1660-72

16. Sakamuro, D., Sabbatini, P., White, E., and Prendergast, G. C. (1997) *Oncogene* **15**(8), 887-98

17. Zhu, J., Jiang, J., Zhou, W., Zhu, K., and Chen, X. (1999) *Oncogene* **18**(12), 2149-55

18. Venot, C., Maratrat, M., Dureuil, C., Conseiller, E., Bracco, L., and Debussche, L. (1998) *Embo J* **17**(16), 4668-79

19. Caelles, C., Helmberg, A., and Karin, M. (1994) *Nature* **370**(6486), 220-3

20. Wagner, A. J., Kokontis, J. M., and Hay, N. (1994) *Genes Dev* **8**(23), 2817-30

21. Haupt, Y., Rowan, S., Shaulian, E., Vousden, K. H., and Oren, M. (1995) *Genes Dev* **9**(17), 2170-83

22. Chen, X., Ko, L. J., Jayaraman, L., and Prives, C. (1996) *Genes Dev* **10**(19), 2438-51

23. Liu, L., Scolnick, D. M., Trievel, R. C., Zhang, H. B., Marmorstein, R., Halazonetis, T. D., and Berger, S. L. (1999) *Mol Cell Biol* **19**(2), 1202-9

24. Gu, W., and Roeder, R. G. (1997) *Cell* **90**(4), 595-606

25. Sakaguchi, K., Herrera, J. E., Saito, S., Miki, T., Bustin, M., Vassilev, A., Anderson, C. W., and Appella, E. (1998) *Genes Dev* **12**(18), 2831-41

26. Hupp, T. R., Meek, D. W., Midgley, C. A., and Lane, D. P. (1992) *Cell* **71**(5), 875-86

27. Halazonetis, T. D., Davis, L. J., and Kandil, A. N. (1993) *Embo J* **12**(3), 1021-8

28. Selivanova, G., Iotsova, V., Okan, I., Fritsche, M., Strom, M., Groner, B., Grafstrom, R. C., and Wiman, K. G. (1997) *Nat Med* **3**(6), 632-8

29. Hupp, T. R., Sparks, A., and Lane, D. P. (1995) *Cell* **83**(2), 237-45

30. Muller-Tiemann, B. F., Halazonetis, T. D., and Elting, J. J. (1998) *Proc Natl Acad Sci U S A* **95**(11), 6079-84

31. Selivanova, G., Ryabchenko, L., Jansson, E., Iotsova, V., and Wiman, K. G. (1999) *Mol Cell Biol* **19**(5), 3395-402

32. Wang, X. W., Vermeulen, W., Coursen, J. D., Gibson, M., Lupold, S. E., Forrester, K., Xu, G., Elmore, L., Yeh, H., Hoeijmakers, J. H., and Harris, C. C. (1996) *Genes Dev* **10**(10), 1219-32

33. Wang, X. W., Yeh, H., Schaeffer, L., Roy, R., Moncollin, V., Egly, J. M., Wang, Z., Freidberg, E. C., Evans, M. K., Taffe, B. G., and et al. (1995) *Nat Genet* **10**(2), 188-95

34. Blander, G., Kipnis, J., Leal, J. F., Yu, C. E., Schellenberg, G. D., and Oren, M. (1999) *J*

Biol Chem **274**(41), 29463-9

35. Spillare, E. A., Robles, A. I., Wang, X. W., Shen, J. C., Yu, C. E., Schellenberg, G. D., and Harris, C. C. (1999) *Genes Dev* **13**(11), 1355-60

36. Zhou, X., Wang, X. W., Xu, L., Hagiwara, K., Nagashima, M., Wolkowicz, R., Zurer, I., Rotter, V., and Harris, C. C. (1999) *Cancer Res* **59**(4), 843-8

37. Gossen, M., and Bujard, H. (1992) *Proc Natl Acad Sci U S A* **89**(12), 5547-51

38. Attardi, L. D., Lowe, S. W., Brugarolas, J., and Jacks, T. (1996) *Embo J* **15**(14), 3693-701

39. Pietenpol, J. A., Tokino, T., Thiagalingam, S., el-Deiry, W. S., Kinzler, K. W., and Vogelstein, B. (1994) *Proc Natl Acad Sci U S A* **91**(6), 1998-2002

40. Sabbatini, P., Lin, J., Levine, A. J., and White, E. (1995) *Genes Dev* **9**(17), 2184-92

41. Miyashita, T., Krajewski, S., Krajewska, M., Wang, H. G., Lin, H. K., Liebermann, D. A., Hoffman, B., and Reed, J. C. (1994) *Oncogene* **9**(6), 1799-805

42. Wu, G. S., Burns, T. F., McDonald, E. R., 3rd, Jiang, W., Meng, R., Krantz, I. D., Kao, G., Gan, D. D., Zhou, J. Y., Muschel, R., Hamilton, S. R., Spinner, N. B., Markowitz, S., Wu, G., and el-Deiry, W. S. (1997) *Nat Genet* **17**(2), 141-3

43. Polyak, K., Xia, Y., Zweier, J. L., Kinzler, K. W., and Vogelstein, B. (1997) *Nature* **389**(6648), 300-5

44. Murphy, M., Hinman, A., and Levine, A. J. (1996) *Genes Dev* **10**(23), 2971-80

45. Gottlieb, T. M., and Oren, M. (1996) *Biochim Biophys Acta* **1287**(2-3), 77-102

46. el-Deiry, W. S. (1998) *Semin Cancer Biol* **8**(5), 345-57

47. Chen, J., Willingham, T., Shuford, M., Bruce, D., Rushing, E., Smith, Y., and Nisen, P. D. (1996) *Oncogene* **13**(7), 1395-403

48. Chen, Y. Q., Cipriano, S. C., Arenkiel, J. M., and Miller, F. R. (1995) *Cancer Res* **55**(20), 4536-9

49. el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993) *Cell* **75**(4), 817-25

50. Sekiguchi, T., and Hunter, T. (1998) *Oncogene* **16**(3), 369-80

51. Sheikh, M. S., Rochefort, H., and Garcia, M. (1995) *Oncogene* **11**(9), 1899-905

52. Brugarolas, J., Chandrasekaran, C., Gordon, J. I., Beach, D., Jacks, T., and Hannon, G. J. (1995) *Nature* **377**(6549), 552-7

53. Deng, C., Zhang, P., Harper, J. W., Elledge, S. J., and Leder, P. (1995) *Cell* **82**(4), 675-84

54. Molinari, M., Okorokov, A. L., and Milner, J. (1996) *Oncogene* **13**(10), 2077-86

55. Pariat, M., Carillo, S., Molinari, M., Salvat, C., Debussche, L., Bracco, L., Milner, J., and Piechaczyk, M. (1997) *Mol Cell Biol* **17**(5), 2806-15

56. Kubbutat, M. H., and Vousden, K. H. (1997) *Mol Cell Biol* **17**(1), 460-8

57. Mee, T., Okorokov, A. L., Metcalfe, S., and Milner, J. (1999) *Br J Cancer* **81**(2), 212-8

58. Okorokov, A. L., and Milner, J. (1997) *Oncol Res* **9**(6-7), 267-73

59. Kubbutat, M. H., Jones, S. N., and Vousden, K. H. (1997) *Nature* **387**(6630), 299-303

60. Oliner, J. D., Pietenpol, J. A., Thiagalingam, S., Gyuris, J., Kinzler, K. W., and Vogelstein, B. (1993) *Nature* **362**(6423), 857-60

61. Honda, R., Tanaka, H., and Yasuda, H. (1997) *FEBS Lett* **420**(1), 25-7

62. Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997) *Nature* **387**(6630), 296-9

63. Kubbutat, M. H., Ludwig, R. L., Ashcroft, M., and Vousden, K. H. (1998) *Mol Cell Biol* **18**(10), 5690-8

64. Swisher, S. G., Roth, J. A., Nemunaitis, J., Lawrence, D. D., Kemp, B. L., Carrasco, C. H., Connors, D. G., El-Naggar, A. K., Fossella, F., Glisson, B. S., Hong, W. K., Khuri, F. R., Kurie, J. M., Lee, J. J., Lee, J. S., Mack, M., Merritt, J. A., Nguyen, D. M., Nesbitt, J. C., Perez-Soler, R., Pisters, K. M., Putnam, J. B., Jr., Richli, W. R., Savin, M., Waugh, M. K., and et al. (1999) *J Natl Cancer Inst* **91**(9), 763-71

65. Clayman, G. L., el-Naggar, A. K., Lippman, S. M., Henderson, Y. C., Frederick, M., Merritt, J. A., Zumstein, L. A., Timmons, T. M., Liu, T. J., Ginsberg, L., Roth, J. A., Hong, W. K., Bruso, P., and Goepfert, H. (1998) *J Clin Oncol* **16**(6), 2221-32

Figure legends

Fig. 1. The activity of activation domain 2 is necessary for inducing apoptosis. (A) Levels of p53, p21 and actin were assayed by Western blot analysis in cell lines as shown above the blots. Cell extracts were prepared from uninduced cells (-) and cells induced (+) to express p53. HA-tagged p53 was detected with 12CA5 antibody. p53 was detected with anti-p53 monoclonal antibody Pab240. p21 was detected with anti-p21 monoclonal antibody (Ab-1). Actin was detected with anti-actin polyclonal antibody. (B, C) Growth rates of p53(AD2$^-$)-6 and p53(AD2$^-$ΔBD)-9 cells in the absence (□) or presence (◊) of p53 over a 5-day period. (D, E) DNA content was quantified by propidium iodide staining of fixed cells that were uninduced (- p53) or induced (+ p53) to express p53(AD2$^-$). (F, G) Apoptotic cells were quantified by propidium iodide-annexin V staining of cells that were uninduced (- p53) or induced (+ p53) to express p53(AD2$^-$).

Fig. 2. The activity for cell cycle arrest, but not apoptosis, was partially retained in p53(ΔAD2), p53(ΔAD2ΔBD), and p53(ΔPRDΔBD). (A, C, E) Levels of p53, p21 and actin were assayed by Western blot analysis in cell lines as shown in the absence (-) or presence (+) of p53. Antibodies used were as in Fig. 1. (B, D, F) Growth rates of p53(ΔAD2)-23, p53(ΔAD2ΔBD)-14, and p53(ΔPRDΔBD)-2 cells in the absence (□) or presence (◊) of p53 over a 5-day period.

Fig. 3. The C-terminal basic domain is not necessary for apoptosis when activation domain 1 is absent. (A) Levels of p53, p21 and actin were assayed by Western blot analysis in cell lines as shown in the absence (-) or presence (+) of p53. Antibodies used were as in Fig. 1. (B and C) Growth rates of p53(ΔAD1ΔBD)-6 and p53(ΔAD1ΔBD)-7 cells in the absence (□) or presence (◊) of p53 over a 5-day period. (D, E) DNA content was quantified by propidium iodide staining of fixed cells that were uninduced (- p53) or induced (+ p53) to express p53(ΔAD1ΔBD). (F, G) Apoptotic cells were quantified by propidium iodide-annexin V staining of cells that were uninduced (- p53) or induced (+ p53) to express p53(ΔAD1ΔBD).

Fig. 4. p53(ΔAD1ΔBD) is capable of inducing both cell cycle arrest and apoptosis in

MCF7 cells. (A) Levels of p53, p21 and actin were assayed by Western blot analysis in cell lines as shown in the absence (-) or presence (+) of p53. Antibodies used were as in Fig. 1. (B and C) Growth rates of MCF7-p53-24 and MCF7-p53(ΔAD1ΔBD)-15 cells in the absence (□) or presence (◊) of p53 over a 5-day period. (D, E, H, I) DNA content was quantified by propidium iodide staining of fixed cells that were uninduced (- p53) or induced (+ p53) to express p53 (D, E) or p53(ΔAD1ΔBD) (H, I). (F, G, J, K) Apoptotic cells were quantified by propidium iodide-annexin V staining of cells that were uninduced (- p53) or induced (+ p53) to express p53 (F, G) or p53(ΔAD1ΔBD) (J, K).

Fig. 5. At least two of the three domains, that is, activation domain 1, activation domain 2, and the proline-rich domain, are required for inducing cell cycle arrest. Levels of p53, p21 and actin were assayed by Western blot analysis in cell lines as shown in the absence (-) or presence (+) of p53(ΔAD1AD2ΔBD) (A), p53(ΔAD1ΔAD2ΔBD) (B), p53(ΔAD1ΔPRDΔBD) (C), p53(ΔAD1ΔPRD) (D), p53(ΔAD2ΔPRD) (E, lanes 3-10), and p53(ΔAD2ΔPRDΔBD) (E, lanes 11-16). Antibodies used were as in Fig. 1.

Fig. 6. Regulation of *p21* and *BAX* by p53 mutants. A Northern blot was prepared using total RNAs isolated from uninduced cells (-) or cells induced (+) to express wild-type p53 or various p53 mutants as shown above the blot. The blot was probed with cDNAs derived from the *p21, BAX,* and *GADPH* genes, respectively. After normalization to the amount of *GAPDH* transcripts, the levels of induction by wild-type p53 or various p53 mutants were quantified by PhosphorImager and are shown below the blot.

Fig. 7. A model of apoptosis for p53 functional domains.

Table 1. p53 domain and activity

	Domain					Activity			
	AD1[a]	AD2[a]	PRD[a]	DBD+NLS+TD/NES[a]	BD[a]	p21[b]	arrest[c]	death[d]	Ref
Wild-type						+++	+++	+++	e
p53(AD1$^-$)[a]						$^-$/+	+/−	++	e
p53(ΔBD)[a]						++	++	−	e
p53(AD1$^-$ΔBD)						−	−	−	e
p53(ΔAD1)						$^-$/+	++	+++	f
p53(ΔAD1AD2$^-$)		XX				−	−	−	f
p53(ΔAD1ΔAD2)						−	−	−	f
p53(AD1$^-$AD2$^-$)		XX				−	−	−	f
p53(ΔPRD)						++	+++	−	g
p53(AD2$^-$)[a]		XX				+/−	+++	+/−	this study
p53(AD2$^-$ΔBD)		XX				+/−	+	−	this study
p53(ΔAD2)						−	+	+/−	this study
p53(ΔAD2ΔBD)						−	+	−	this study
p53(ΔPRDΔBD)						+/−	+	−	this study
p53(ΔAD1ΔBD)						+	+++	+++	this study
p53(ΔAD1AD2$^-$ΔBD)		XX				−	−	−	this study
p53(ΔAD1ΔAD2ΔBD)						−	−	−	this study
p53(ΔAD1ΔPRDΔBD)						−	−	−	this study
p53(ΔAD1ΔPRD)						−	−	−	this study
p53(ΔAD2ΔPRD)						−	−	−	this study
p53(ΔAD2ΔPRDΔBD)						−	−	−	this study

[a] AD1, activation domain 1 within residues 1-42
AD2, activation domain 2 within residues 43-63
PRD, the proline-rich domain within residues 64-92
BD, the C-terminal basic domain within residues 364-393
TD, the tetramerization domain within residues 334-356
DBD, the DNA binding domain within residues 100-300
NLS, the nuclear localization signal within residues 316-325
AD1$^-$, a double point mutation of L22Q and W23S
AD2$^-$, a double point mutation of W53Q and F54S
Δ, deletion

[b] The ability of p53 to induce p21 was measured by Western and Northern blot analyses

[c] Arrest was measured by DNA histogram analysis

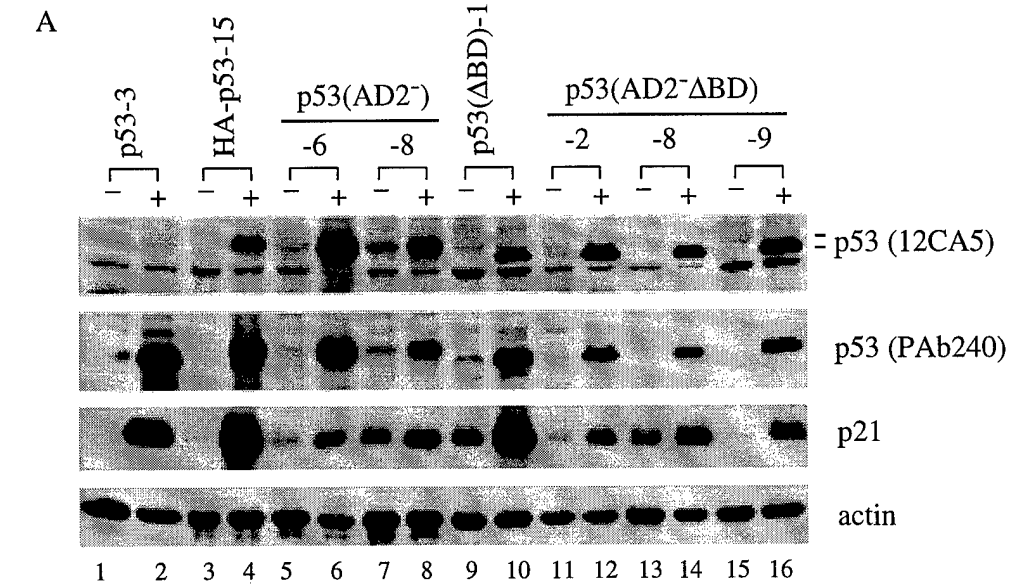
[d] Death was measured by trypan blue dye exclusion and annexin V staining assays and DNA histogram analysis

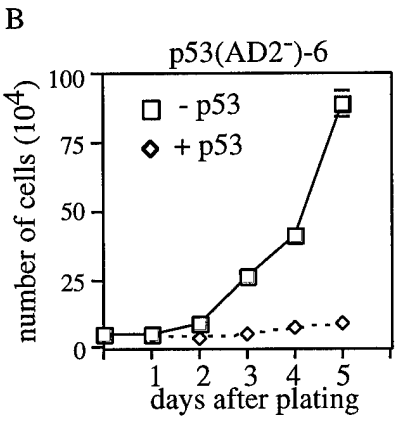
[e] Chen, X., Ko, L.J., Jayaraman, L. & Prives, C. (1996b). *Genes Dev*, **10,** 2438-2451

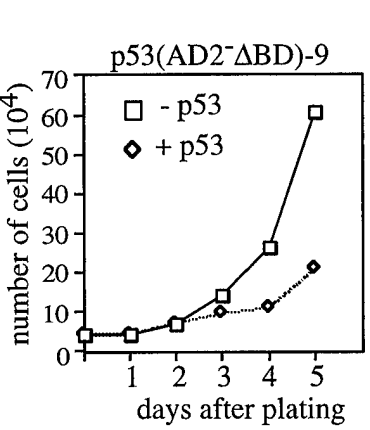
[f] Zhu, J., Zhou, W., Jiang, J. & Chen, X. (1998). *J Biol Chem*, **273,** 13030-13036

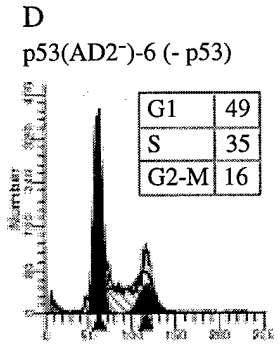
[g] Zhu, J., Jiang, J., Zhou, W., Zhu, K. & Chen, X. (1999). *Oncogene*, **18,** 2149-2155

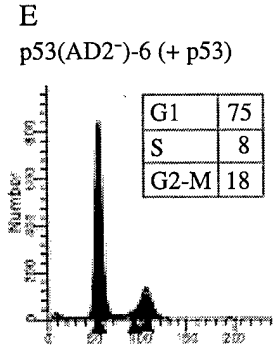
Fig. 1

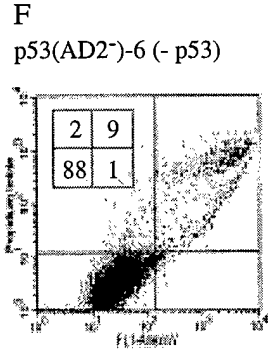

A
p53-3
HA-p53-15
p53(AD2⁻)
-6
-8
p53(ΔBD)-1
p53(AD2⁻ΔBD)
-2
-8
-9
p53 (12CA5)
p53 (PAb240)
p21
actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16


B
p53(AD2⁻)-6
number of cells (10⁴)
- p53
+ p53
days after plating


C
p53(AD2⁻ΔBD)-9
number of cells (10⁴)
- p53
+ p53
days after plating


D
p53(AD2⁻)-6 (- p53)
G1 49
S 35
G2-M 16


E
p53(AD2⁻)-6 (+ p53)
G1 75
S 8
G2-M 18


F
p53(AD2⁻)-6 (- p53)
2 9
88 1

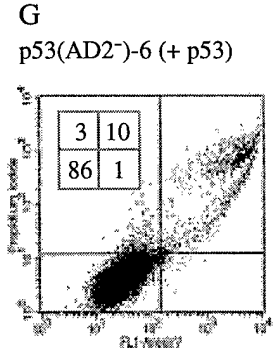

G
p53(AD2⁻)-6 (+ p53)
3 10
86 1

Fig. 2

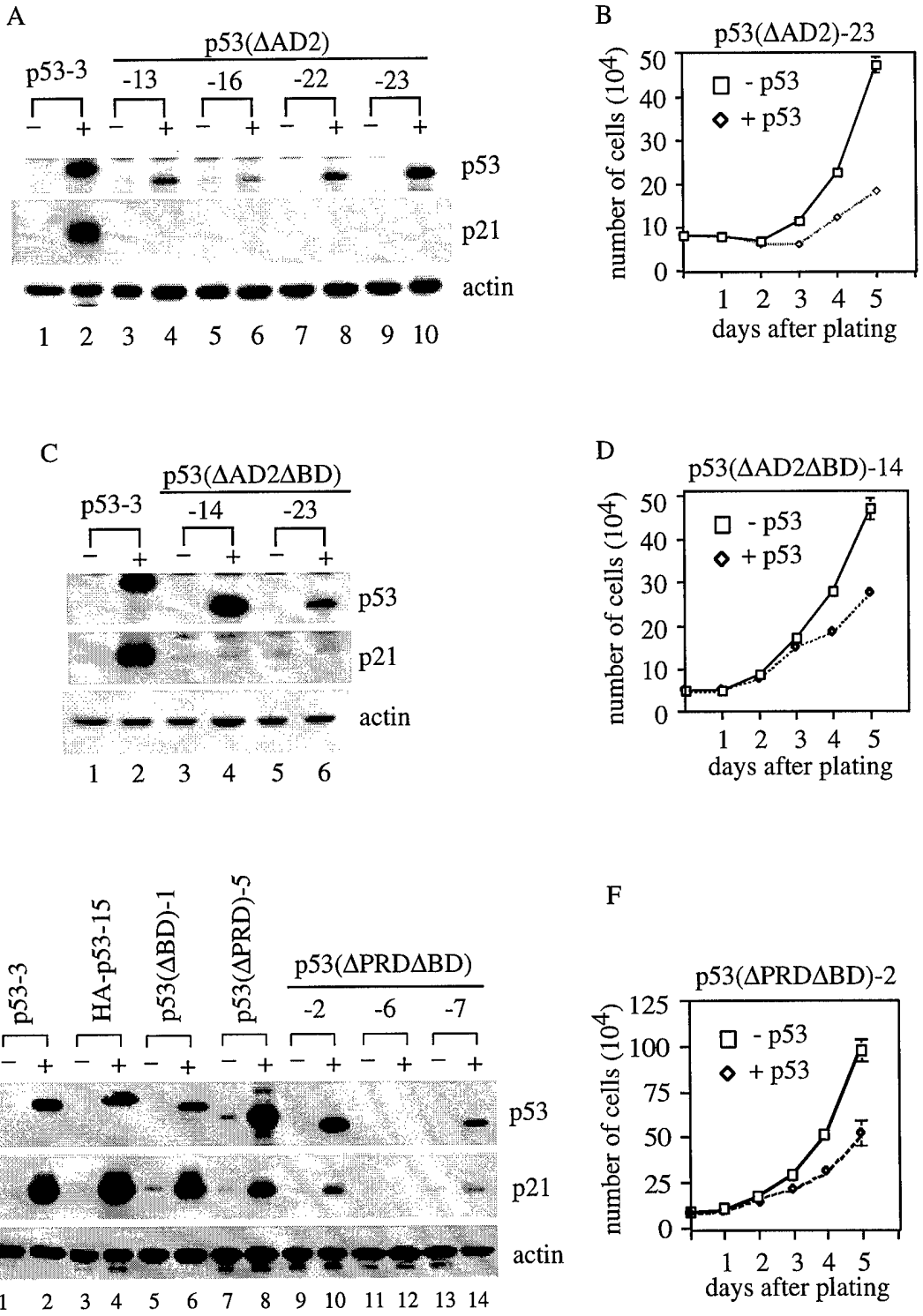
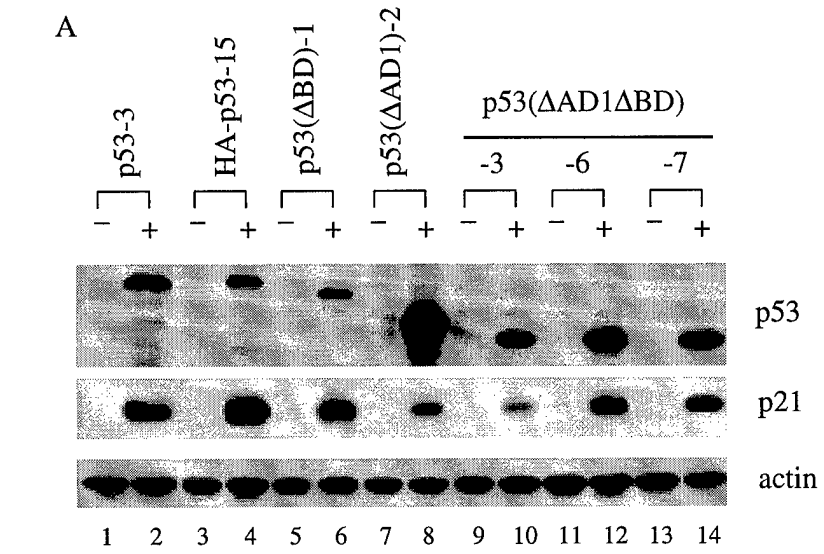
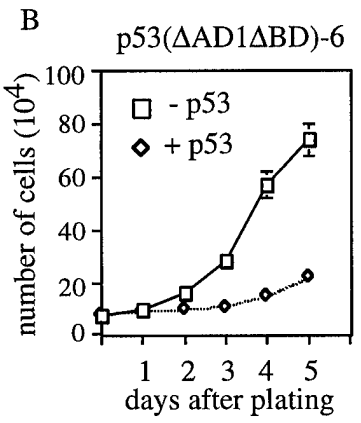
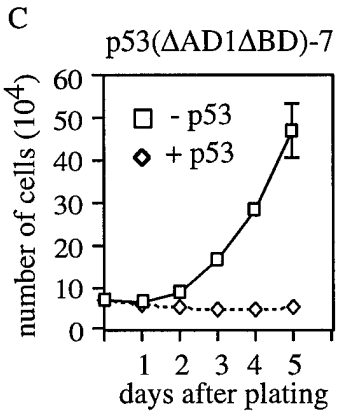
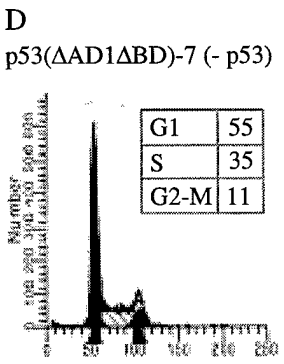

A
p53(ΔAD2)
p53-3
-13
-16
-22
-23
p53
p21
actin
1 2 3 4 5 6 7 8 9 10
B
p53(ΔAD2)-23
- p53
+ p53
number of cells (10^4)
days after plating
C
p53(ΔAD2ΔBD)
p53-3
-14
-23
p53
p21
actin
1 2 3 4 5 6
D
p53(ΔAD2ΔBD)-14
- p53
+ p53
number of cells (10^4)
days after plating
E
p53-3
HA-p53-15
p53(ΔBD)-1
p53(ΔPRD)-5
p53(ΔPRDΔBD)
-2
-6
-7
p53
p21
actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14
F
p53(ΔPRDΔBD)-2
- p53
+ p53
number of cells (10^4)
days after plating

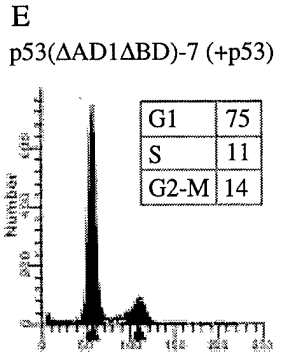
Fig. 3

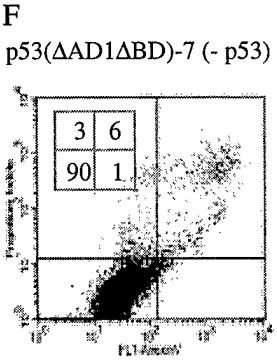

A
p53-3
HA-p53-15
p53(ΔBD)-1
p53(ΔAD1)-2
p53(ΔAD1ΔBD)
-3
-6
-7
p53
p21
actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14


B
p53(ΔAD1ΔBD)-6
number of cells (10^4)
- p53
+ p53
days after plating


C
p53(ΔAD1ΔBD)-7
number of cells (10^4)
- p53
+ p53
days after plating


D
p53(ΔAD1ΔBD)-7 (- p53)
G1 55
S 35
G2-M 11


E
p53(ΔAD1ΔBD)-7 (+p53)
G1 75
S 11
G2-M 14


F
p53(ΔAD1ΔBD)-7 (- p53)
3 6
90 1

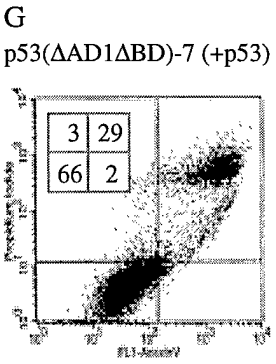

G
p53(ΔAD1ΔBD)-7 (+p53)
3 29
66 2

Fig. 4

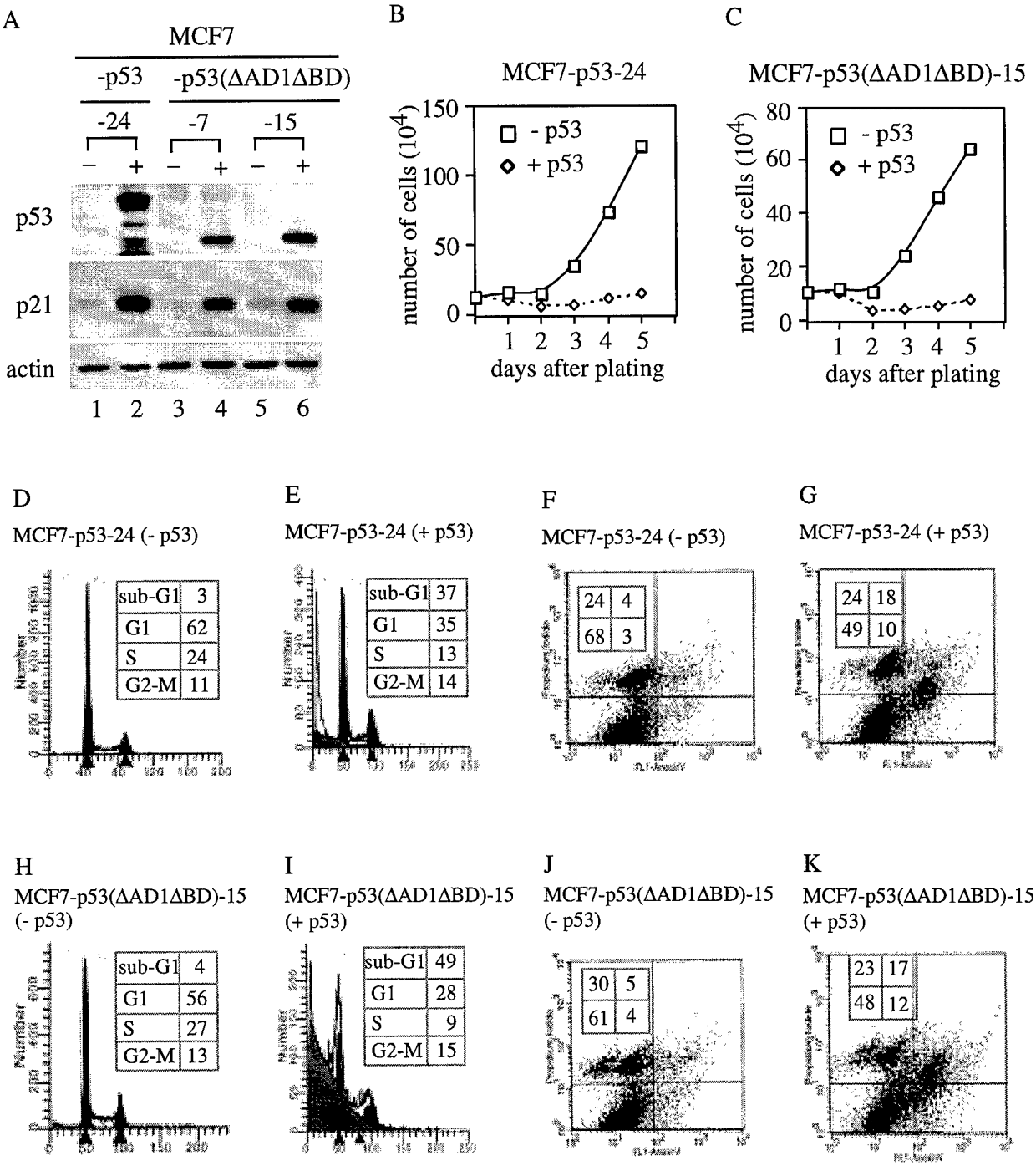

A
MCF7
-p53
-p53(ΔAD1ΔBD)
-24
-7
-15
– + – + – +
p53
p21
actin
1 2 3 4 5 6
B
MCF7-p53-24
number of cells (10^4)
- p53
+ p53
days after plating
C
MCF7-p53(ΔAD1ΔBD)-15
number of cells (10^4)
- p53
+ p53
days after plating
D
MCF7-p53-24 (- p53)
sub-G1 3
G1 62
S 24
G2-M 11
E
MCF7-p53-24 (+ p53)
sub-G1 37
G1 35
S 13
G2-M 14
F
MCF7-p53-24 (- p53)
24 4
68 3
G
MCF7-p53-24 (+ p53)
24 18
49 10
H
MCF7-p53(ΔAD1ΔBD)-15
(- p53)
sub-G1 4
G1 56
S 27
G2-M 13
I
MCF7-p53(ΔAD1ΔBD)-15
(+ p53)
sub-G1 49
G1 28
S 9
G2-M 15
J
MCF7-p53(ΔAD1ΔBD)-15
(- p53)
30 5
61 4
K
MCF7-p53(ΔAD1ΔBD)-15
(+ p53)
23 17
48 12

Fig. 5

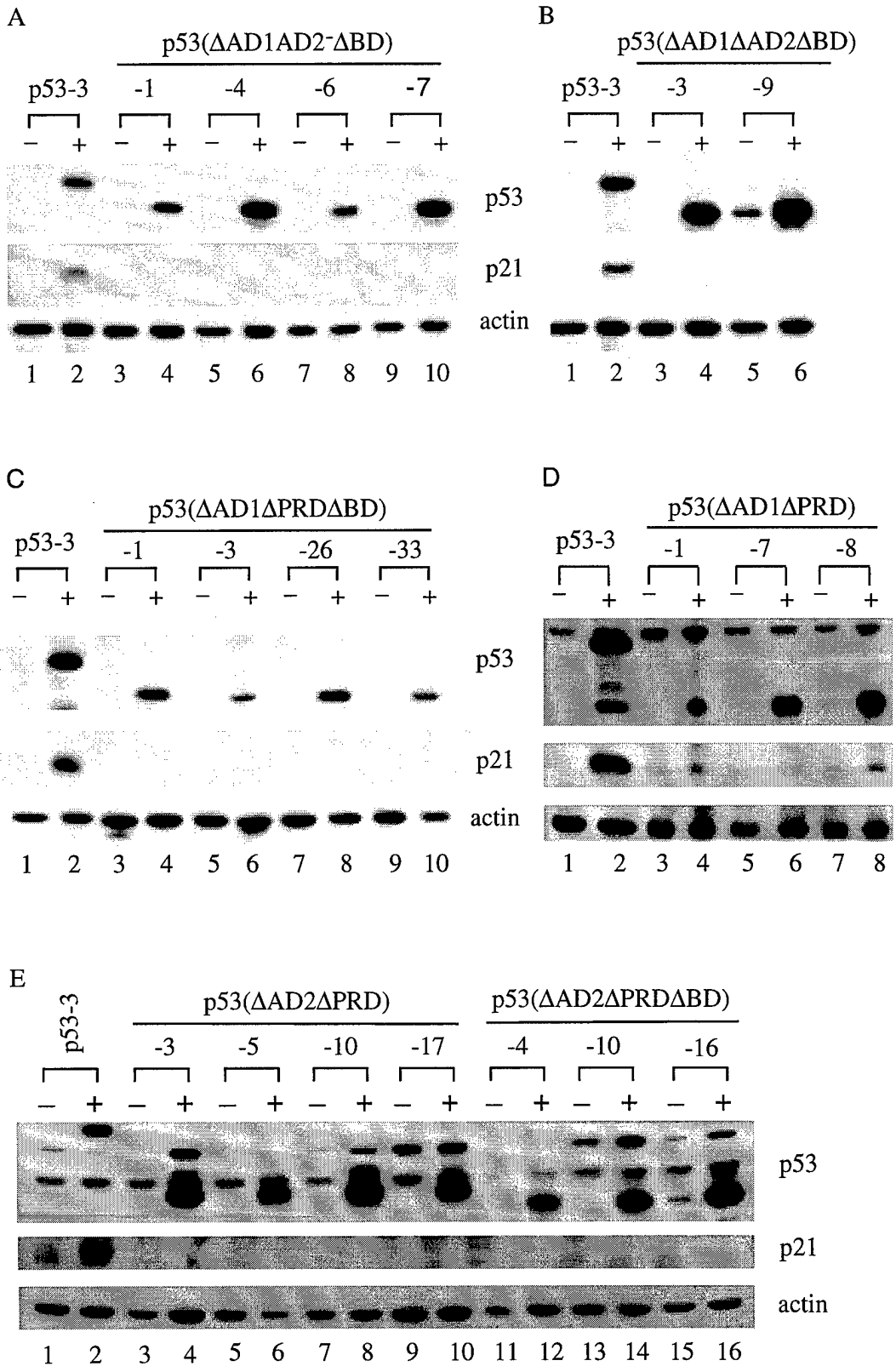

A
p53(ΔAD1AD2ΔBD)
p53-3
-1
-4
-6
-7
p53
p21
actin
1 2 3 4 5 6 7 8 9 10
B
p53(ΔAD1ΔAD2ΔBD)
p53-3
-3
-9
p53
p21
actin
1 2 3 4 5 6
C
p53(ΔAD1ΔPRDΔBD)
p53-3
-1
-3
-26
-33
p53
p21
actin
1 2 3 4 5 6 7 8 9 10
D
p53(ΔAD1ΔPRD)
p53-3
-1
-7
-8
p53
p21
actin
1 2 3 4 5 6 7 8
E
p53-3
p53(ΔAD2ΔPRD)
-3
-5
-10
-17
p53(ΔAD2ΔPRDΔBD)
-4
-10
-16
p53
p21
actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Fig. 6

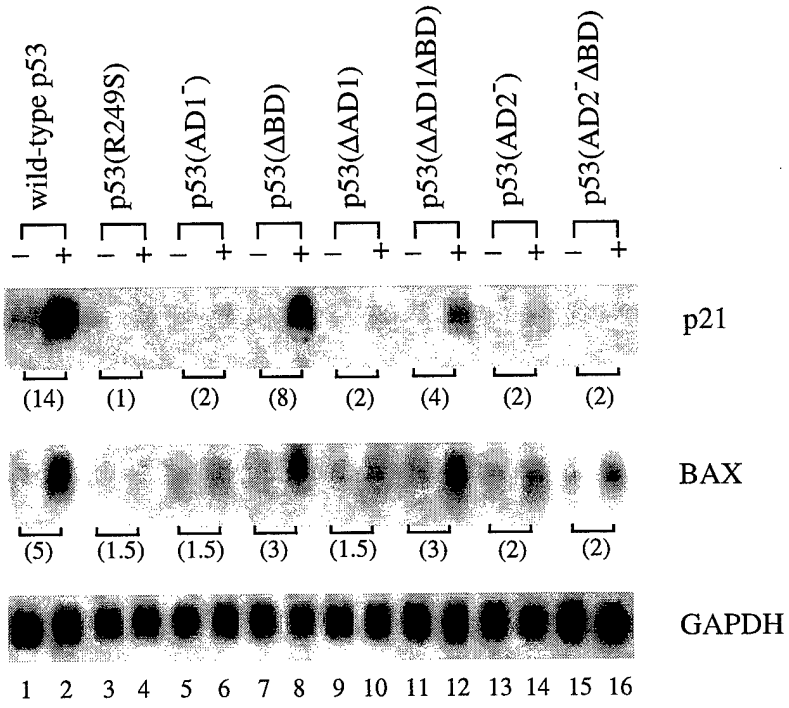
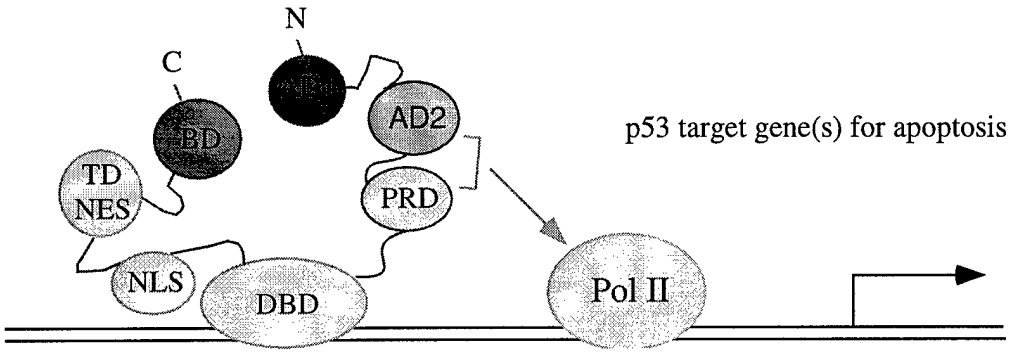

wild-type p53
p53(R249S)
p53(AD1⁻)
p53(ΔBD)
p53(ΔAD1)
p53(ΔAD1ΔBD)
p53(AD2⁻)
p53(AD2⁻ΔBD)
– + – + – + – + – + – + – + – +
p21
(14) (1) (2) (8) (2) (4) (2) (2)
BAX
(5) (1.5) (1.5) (3) (1.5) (3) (2) (2)
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Fig. 7


N
C
AD2
BD
TD
NES
PRD
NLS
DBD
Pol II
p53 target gene(s) for apoptosis

The American Association for Cancer Research 91th annual meeting
Abstract #3958

Definition of the p53 functional domains necessary for inducing apoptosis.

Jianhui Zhu, Shunzhen Zhang, Jieyuan Jiang, and Xinbin Chen. Medical College of Georgia, Augusta, GA 30912.

It has been well established that p53 contains several functional domains necessary for inducing cell cycle arrest and apoptosis. The C-terminal basic domain (BD), within residues 364-393, and the proline-rich domain (PRD), within residues 64-91, are required for apoptosis. In addition, the activation domain II (AD2), within residues 43-63, is necessary for apoptosis when the N-terminal AD1, within residues 1-42, is deleted (ΔAD1) or mutated ($AD1^-$). Here we showed that AD2 mutation at residues 53-54 ($AD2^-$) abrogates the apoptotic activity but not cell cycle arrest. We also found that the activity of other mutants (p53(ΔAD2), p53($AD2^-$ΔBD), p53(ΔAD2ΔBD), p53(ΔPRDΔBD), and p53(ΔAD1ΔPRD) in apoptosis is abrogated but in arrest severely diminished. Interestingly, deletion of the N-terminal AD1 alleviates the requirement of the C-terminal BD for apoptosis. Thus, we have generated a very small but potent p53(ΔAD1ΔBD) molecule. Furthermore, when twenty-two cellular genes, e.g., p21, BAX, KILLER/DR5, IGFBP3, and PIGs, were screened for induction by various p53 mutants, we found that none of these genes are necessary and/or sufficient for inducing apoptosis. Nevertheless, we found that G1 arrest can be mediated by other undetermined p53 target gene(s) in addition to p21. Finally, we propose that AD2 and PRD form an activation domain for inducing pro-apoptotic genes. The carboxyl BD is required for this activation domain to be competent for transactivation.

DOD Era of Hope Meeting
Poster # B-77

DEFINITION OF THE P53 FUNCTIONAL DOMAINS NECESSARY FOR INDUCING APOPTOSIS

Xinbin Chen

Institute of Molecular Medicine and Genetics,
Medical College of Georgia, Augusta, GA 30912

E-mail: xchen@mail.mcg.edu

The ability of p53 to induce apoptosis requires the DNA binding activity; however, a double point mutation at residues 22-23 in the activation domain 1 within residues 1-42 ($AD1^-$) can still induce apoptosis. Here we showed that deletion of the N-terminal 23 amino acids compromises, but does not abolish p53 induction of apoptosis. Surprisingly, p53(ΔAD1), which lacks the N-terminal 42 amino acids and the previously defined activation domain 1, retains the ability to induce apoptosis. A more extensive deletion, which eliminates the N-terminal 63 amino acids, renders p53 completely inert in mediating apoptosis. In addition, we found that both p53(ΔAD1) and p53($AD1^-$) can induce a subset of cellular p53 target genes. These data suggest that within residues 43 to 63 lies an apoptotic domain as well as another activation domain, AD2.

p53 contains a proline-rich domain (PRD), located within residues 60-90, which is necessary for efficient growth suppression. We found that p53(ΔPRD), which lacks the proline-rich domain, is capable of inducing cell cycle arrest but not apoptosis, while p53($AD1^-$/ΔPRD), which contains a double point mutation in the activation domain 1 as well as deletion of the proline-rich domain, completely loses its activity. In addition, we found that, while p53(ΔPRD) is capable of activating several transiently transfected promoters, e.g., p21 and MDM2, its ability to induce several target genes, e.g., p21 and MDM2, is diminished. These results suggest that the proline-rich domain may play a role in chromatin remodeling, which counteracts chromatin-mediated repression for some of the endogenous p53 target genes.

To further analyze AD1, AD2, and PRD, we found that a double point mutation at residues 53-54 ($AD2^-$) or deletion of AD2 (ΔAD2) abrogates the apoptotic activity but not cell cycle arrest. Interestingly, deletion of AD1 alleviates the requirement of the C-terminal basic domain within residues 364-393 for apoptosis. Furthermore, when twenty-two cellular genes, e.g., p21, BAX, KILLER/DR5, IGFBP3, and PIGs, were screened for induction by various p53 mutants, we found that none of these genes are necessary and/or sufficient for inducing apoptosis. Finally, we propose that AD2 and PRD form an activation domain for inducing pro-apoptotic genes. The carboxyl basic domain is required for this activation domain to be competent for transactivation.

The 10th p53 workshop
Poster #32

Definition of the p53 functional domains necessary for inducing cell cycle arrest and apoptosis

Xinbin Chen, Jianhui Zhu, Shunzhen Zhang, and Jieyuan Jiang

Institute of Molecular Medicine and Genetics, Medical College of Georgia, Augusta, GA 30912

The p53 protein contains several functional domains that are necessary for inducing cell cycle arrest and apoptosis. The C-terminal basic domain (BD), within residues 364-393, and the proline-rich domain (PRD), within residues 64-91, are required for apoptotic activity. In addition, the activation domain II (AD2), within residues 43-63, is necessary for apoptotic activity when the N-terminal AD1, within residues 1-42, is deleted (ΔAD1) or mutated ($AD1^-$). Here we found that AD2 mutation at residues 53-54 ($AD2^-$) abrogates the apoptotic activity but has no significant effect on cell cycle arrest. We also found that p53($AD2^-$ΔBD), p53(ΔAD2), p53(ΔAD2ΔBD), and p53(ΔPRDΔBD) are inert in inducing apoptosis but still active in inducing cell cycle arrest albeit to a lesser extent than wild-type p53. Interestingly, deletion of the N-terminal AD1 alleviates the requirement of the C-terminal BD for apoptotic activity. Thus, we have generated a very small but potent p53(ΔAD1ΔBD) molecule. In addition, we found that at least two of the three domains, that is, AD1, AD2 and PRD, are required for inducing cell cycle arrest. Furthermore, we found that G1 arrest can be mediated by other undetermined p53 target gene(s) in addition to p21. Taken together, we propose that AD2 and PRD form an activation domain for inducing pro-apoptotic genes. The carboxyl BD is required for this activation domain to be competent for transactivation.